Thesis for the Degree of Master of Science

Endocrine Mechanism of Sex Change Process in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

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Department of Marine Bioscience and Environment

The Graduate School

Korea Maritime University

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Endocrine Mechanism of Sex Change Process in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

Advisor: Prof. Cheol Young Choi

by

Kwang Wook An

A dissertation submitted in partial fulfillment of the requirements for the degree of

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A dissertation

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요 약

어류의 번식 조절은 생식소자극호르몬 방출호르몬 (gonadotropin-releasing hormone, GnRH), 생식소자극호르몬 (gonadotropin, GTH), 생식소호르몬 및 신경호르몬을 포함한 많은 인자들이 상호작용하여 이루어진다. 본 연구에서는 웅성선숙형 해산어류인 감성돔, *Acanthopagrus schlegeli*의 성 전환 메커니즘을 밝히기 위해 뇌하수체와 생식소로부터 성 전환 관련 유전자인 GnRH isoforms (salmon GnRH, sGnRH, GenBank accession no. EU117212; seabream GnRH, sbGnRH, EU099997; chicken GnRH-II, cGnRH-II, EU099996), estrogen receptor β2 (ERβ2, EU346949), GTH subunits (GTHα, EU605275; follicle-stimulating hormone, FSHβ, AY921613; luteinizing hormone, LHβ, EU605276) 및 GTH receptors (FSHR, EU095497; LHR, EU339125)를 클로닝 하였고, 성 전환 과정 동안 이들 유전자의 발현량 변화를 비교하였다.

감성돔의 성 전환 과정을 생식소 발달 단계에 따라 immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary 및 mature ovary로 총 7

단계로 구분하였으며, 각 발달 단계별 뇌하수체와 생식소에서 이들 유전자의 발현량은 quantitative real-time PCR (QPCR)을 이용하여 분석하였다. sGnRH, sbGnRH 및 cGnRH-II 발현은 성숙한 수컷의 정소와 암컷의 난소에서 높게 발현되었다. 또한 ERα와 ERβ1은 성숙한 수컷의 정소와 암컷의 난소에서 높은 발현이 관찰되었으나, ERβ2는 성숙한 암컷의 난소에서만 높게 발현되었다. 뇌하수체 GTHα, FSHβ 및 LHβ 발현은 성숙한 수컷과 암컷에서 높게 관찰되었으며, 이들의 수용체인 FSHR과 LHR 발현 또한 성숙한 수컷과 암컷에서 높게 발현되었다.

난모세포의 발달은 조직학적 분석을 통하여 관찰되었는데, 성숙한 암컷의 난소에서 난황을 가진 난모세포가 다수 관찰되었으며, 이 시기 동안 혈장 E_2의 농도도 매우 높게 증가되었다.

본 연구에서는, 감성돔에서 GnRH와 E_2의 역할을 알아보기 위하여, 미성숙 감성돔에 GnRH analogue (GnRHa)와 E_2를 주입하여 GTH subunits와 GTH receptors mRNA의 발현량 및 혈장 E_2 농도의 변화를 관찰하였다. GnRHa와 E_2 처리 후, GTH subunits와 GTH receptors mRNA의 발현량과 혈장 E_2 농도는 증가하는 경향을 나타내었다.

본 연구를 통하여 GnRH와 E_2가 시상하부-뇌하수체-생식소 축의 조절에 중요한 역할을 하고 있다는 사실을 확인할 수 있었으며, GnRH isoforms, ER subunits, GTH subunits 및 GTH receptors는 웅성선숙형 감성돔의 생식소 발달 및 성 전환에 관여하고 있다는 것을 알 수 있었다.

I. General Introduction

The regulation of reproduction in teleost fish is a complex process involving the interaction of a number of factors including gonadotropin -releasing hormone (GnRH), gonadotropin (GTH), gonadal hormones and other neurohormones. GnRH is decapeptide neurohormones responsible for the control and coordination of reproduction in all vertebrates (Kavanough et al., 2008), and is released by the hypothalamus and stimulates the synthesis and release of hypophyseal GTHs; follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The GTHs have two subunits; GTHα, which is common to FSH, LH, and thyroid stimulating hormone, and β subunit which is specific to either FSH or LH. FSH and LH act by binding specific receptors (FSHR and LHR) in the gonad of vertebrates to induce steroidogenesis and gametogenesis (Nagahama et al., 1995). Pituitary FSH regulates both vitellogenesis and spermatogenesis in early developmental stages, and LH stimulates the synthesis and secretion of steroid hormones (e.g. estrogen, progesterone, androgen, and testosterone) from the gonads, thereby regulating ovarian and testicular function in teleosts (Ando and Urano, 2005). Thus, gonadal maturation is primarily regulated by the hypothalamus-pituitary-gonad axis, and GnRH plays a central role in the regulation of gonadal maturation and reproduction in fish and other species.

There are three types of hermaphroditism: (1) protogyny, in which some or all individuals function first as females and later in life function exclusively as males; (2) protandry, in which sex change is from male to

female; and (3) simultaneous hermaphroditism, in which the individual functions at the same time of life as both male and female. Among these types, black porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae), are marine protandrous hermaphrodites that are widely distributed and are of particular interest for commercial aquaculture in parts of Asia including Korea. These fish are functional male for their first 2 years of life, but approximately 70% of black porgy change into females during the third spawning season in their natural environment.

Therefore, the objectives of this study were (1) to investigate the expression of GnRH isoforms, ER subunits, GTH subunits, and GTH receptors transcript during sex-change process, with associated changes in circulating estradiol-17β (E_2) levels in plasma and histological changes of gonads; (2) to test the hypothesis that GnRH and E_2 regulates induction of pituitary FSH and LH, and the subsequent increase in circulating E_2 levels in plasma in immature black porgy; (3) to describe the interaction among these sex-change related genes and hypothalamus-pituitary-gonad axis of black porgy.

II. Experiment 1

Characterization of Estrogen Receptor β2 and Expression of the Estrogen Receptor subtypes α, β1, and β2 in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

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Characterization of estrogen receptor β2 and expression of the estrogen receptor subtypes α, β1, and β2 in the protandrous black porgy (*Acanthopagrus schlegeli*) during the sex change process

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ABSTRACT

Estrogens play an important role in many physiological processes in both female and male vertebrates, mediated by specific nuclear receptor, estrogen receptors (ERs). We have isolated a third ER (ERβ2), which was found to contain 2004 nucleotides including an open reading frame that encodes 667 amino acids. We have also cloned ERα and ERβ1 from the published information (GenBank accession nos. AY074780 and AY074779) and investigated the expression pattern of these ER subtypes in the gonads during gonad sex change of black porgy by quantitative polymerase chain reaction. Maturity stages can be divided into five stages during the sex change process from immature male to female (immature male, mature male, male of mostly testis, male of mostly ovary and mature female). The expression of ERα mRNA was highest in the ovary of mature female, followed by the testis of mature male and testicular portion of mostly testis. ERβ1 expression was higher in the mature testis and ovary than in the gonads of other maturity stages. In contrast to that, ERβ2 was highest in the ovary of mature female, and significantly lower levels of ERβ2 expression were observed in the gonads of the other maturity stages. The present study describes the molecular characterization of ERβ2, and documents the expression changes of three ER subtypes during sex change process of the protandrous black porgy.

1. Introduction

In vertebrates, including fish, the steroid hormone estrogen is essential in reproduction, playing important roles in several aspects of sexual maturation and differentiation that include oogenesis, vitellogenesis, and testicular development. In addition, estrogen has been shown to function in non-reproductive tissues such as the bone, brain, and heart (Ishibashi and Kawashima, 2001). The mechanism of estrogen action is mediated by the nuclear estrogen receptors (ERα and ERβ), which function as ligand-dependent transcription factors that regulate transcription of target genes containing the consensus estrogen response element (ERE) in their promoter regions. Also, estrogens exert its actions through a membrane ER (mER), and the various signaling pathways (Ca^{2+}, cAMP, protein kinase cascades) are rapidly activated and ultimately influence downstream transcription factors (Zhang and Trudeau, 2006).

ERs are part of a large superfamily of ligand-activated nuclear receptors that includes receptors for other steroid hormones and thyroid hormone, in addition to a group of so-called orphan receptors (Mangelsdorf et al., 1995). The members of this superfamily share several common features that can be divided into six distinct domains (Krust et al., 1986; Kumar et al., 1987), including the highly conserved C (DNA-binding domain; DBD) and E (ligand-binding domain; LBD) domains, as well as many variable regions at the N and C termini between the DBD and LBD (the A/B, F and D domains) (Choi and Habibi, 2003).

Sequence alignments support the existence of ERα and ERβ subtypes in fish (Chang et al., 1999; Tchoudakova et al., 1999; Xia et al., 1999; Hawkins et al., 2000; Ma et al., 2000; Pakdel et al., 2000; Patiño et al., 2000; Rogers et al., 2000; Socorro et al., 2000; Choi and Habibi, 2003; Halm et al., 2004), including black porgy (Huang and Chang, 2002). In teleosts, a third ER subtype, called ERβ2 (Tchoudakova et al., 1999; Ma et al., 2000; Menuet et al., 2002) or ERγ (Hawkins et al., 2000; Halm et al., 2004), has been cloned that is genetically distinct from the other two. However, no information is available regarding the sequence or expression pattern of ERβ2 mRNA during the reproductive cycle in black porgy.

The discovery of the various ER subtypes and their presence in the female and male reproductive systems in equivalent amounts has generated great interest regarding their potential functions during sexual differentiation and development. In teleosts, ERs are expressed very early during embryonic development and gonadal differentiation,suggesting an important role of estrogen in sexual

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differentiation in fish (Guiguen et al., 1999; Lassiter et al., 2002). However, a comprehensive study, investigating the expression of all three ER subtypes during sex change associated with histological differences is currently lacking for protandrous hermaphrodite fish.

The black porgy (*Acanthopagrus schlegeli*, Perciformes, Sparidae), a widely distributed marine protandrous hermaphrodite, is of particular interest in commercial aquaculture in various parts of Asia, including Korea. Black porgies are well-known to be functional males for the first 2 years of life, and then begin to change sex during the third year. However, only about 40–50% of black porgies change into females during the third spawning season (Chang et al., 1994).

In this study, we isolated and characterized ERβ2 cDNA and analyzed the mRNA expression of ERα, ERβ1, and ERβ2 in the black porgy to clarify the mechanisms involved in the sexual differentiation and development of this species. We also examined the maturity of the gonads by histological analysis during the sex change process.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature fish (51.0 ± 2.3 g, 1 year), mature male (220 ± 14.2, 2 year), sex changing fish (489.2 ± 11.5 g, 3 years) and female black porgy (948.5 ± 51.6 g, 4 years). Sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were netted and anesthetized in tricaine methanesulfonate (MS-222, Sigma-Aldrich, USA), and decapitated prior to tissue collection. Gonad samples from black porgy at the each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and mature ovary) were removed, immediately frozen in liquid nitrogen and stored at − 80 °C until the total RNA was extracted for analysis.

2.2. Total RNA extraction and reverse transcription (RT)

Total RNA from black porgy at each gonad maturity stages during sex change process were extracted using the TRI REGENT, according to the manufacturer's instructions (Gibco/BRL, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. 3 μg of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at − 20 °C for use in polymerase chain reaction (PCR) and quantitative PCR.

2.3. Identification of ERβ2 cDNA

Primers for ERβ2 were designed using highly conserved regions of gilthead seabream (GenBank accession no. AJ580048) and killifish (AY570923): bpERβ2 forward primer (5′-GGA AGA GAT CAG ACG AGA GG-3′) and bpERβ2 reverse primer (5′-AGC AGC ATG GTG AGG TGT C-3′). PCR amplification was performed using a 2× Taq Premix I (Solgent, Korea) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 54 °C for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. Amplified PCR products were visualized by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea) and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoR I (Fermentas, USA). Based on the plasmid DNA, the ERβ2 cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.4. Rapid amplification of ERβ2 cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from the gonads using the TRI REGENT (Gibco/BRL, USA). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-d$(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T$(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

ERβ2-specific primers were selected from the PCR product obtained by RT-PCR. For the 3′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mmol/L 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mmol/L 3′ RACE gene-specific primer (5′-GAA GCT TCA GAG GGA GGA GTA CGT CTG-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by 1 cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mmol/L 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mmol/L 5′ RACE gene-specific primer (5′-CGC ACT TCA TGC CCA CTT CGT AGC-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis in a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The transformation was conducted as mentioned above.

2.5. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length ERβ2 cDNA from various fishes. Amino acid sequences were aligned using the BioEdit Software (Hall, 1999). Sequences used for comparison and their GenBank accession numbers are as follows: black porgy ERβ2 (bpERb2, EU346949), gilthead seabream ERβ2 (gsERb2, CAE30469), largemouth bass ERβ (lbERb, AAO39210), killifish ERβb (kfERbb, AAU44353), Nile tilapia ERβ2 (ntERb2, ABE73151), Mozambique tilapia ERβ2 (mtERb2, ABV55459), rainbow trout ERβ2 (rtERb2, ABB73309), zebrafish ERβb (zfERbb, AAK16741), goldfish ERβ2 (gfERb2, AAF35170) and human ERβ (hmERb, Q62986). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and analyzed using Mega 3.1 software package (Kumar et al., 2001). The degree of support for internal branches was inferred using bootstrapping (1000 replicates) analysis.

2.6. Quantitative polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative expression of ER subtypes (ERα, ERβ1 and ERβ2) mRNA using total RNA extracted from the gonads of black porgy ($n=3$). Primers for QPCR were designed with reference to the known sequences of black porgy as follows: ERα forward primer (5′-CTA CTA CTC TGC TCC TCT GG-3′), ERα (5′-ATG AAG GGG CTG AGA CGG-3′), ERβ1 forward primer (5′-GCC ATA CCT TTC TAC AGT CC-3′), ERβ1 reverse primer (5′-CCG TGC TGA GGT CGA GCC-3′), ERβ2 forward primer (5′-GTC TGG ATA GGG TTC GTG G-3′), ERβ2 reverse primer (5′-AAG GCA AAT GGC TTC TTG GG-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′) and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). QPCR

amplification was conducted similar to previous work (Nelson et al., 2007), using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and with the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr green and *Taq* polymerase in buffer (10 mM Tris–HCL [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μL. QPCR was carried out as follows: 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin Ct levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from three replicate samples were analyzed with analysis

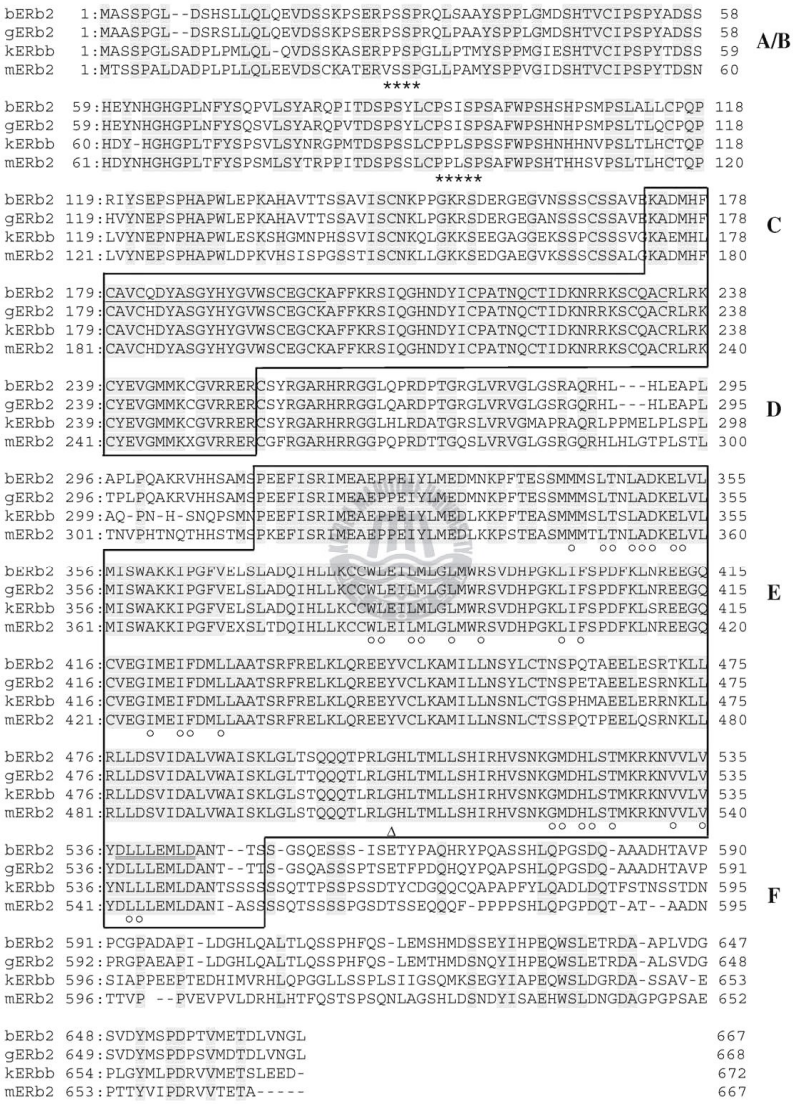

Fig. 1. Comparison of the amino acid sequence of black porgy (*Acanthopagrus schlegeli*) ERβ2, gilthead seabream (*Sparus aurata*) ERβ2, killifish (*Fundulus heteroclitus*) ERβb and Mozambique tilapia (*Oreochromis mossambicus*). ERβ2 was optimally aligned to match identical residues, indicated by the shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The ERβ2 sequences used for alignment are black porgy ERβ2 (bERb2, this paper, EU346949), gilthead seabream ERβ2 (gERb2, AJ580048), killifish ERβb (kERbb, AY570923) and Mozambique tilapia ERβ2 (mERb2, EU140820). ER domains are indicated on the right (C and E boxed in solid line). Motifs shown: putative phosphorylation sites for the MAPK pathway in the A/B domain (*); two conserved zinc-finger motifs in the C domain (underline); amino acids recognized to be involved in dimerization (Δ), ligand interaction (O) and transactivation (AF-2 domain, double underline) in the E domain.

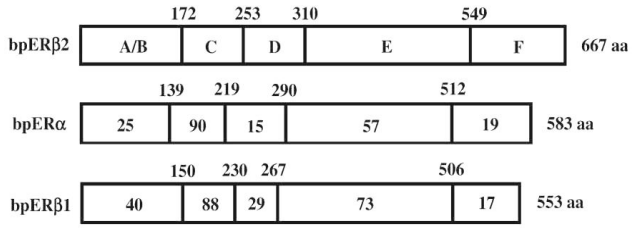

Fig. 2. Domain structure of the bpERβ2 and similarity with bpERα and bpERβ1. The percentage of amino acid identity of each domain relative to the bpERβ2 is indicated within the box representing the corresponding domain.

software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 97.3%, ERα = 97.2%, ERβ1 = 96.0% and ERβ2 = 98.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

2.7. Gonad histology

The gonad tissues from each developmental group (mature testis, testicular and ovarian portions of the sex changing gonad and mature ovary) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin-eosin for observation under a light microscope (Olympus BS50, Japan) and images captured with a digital camera (Olympus DP-50, Japan).

2.8. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Newman Keul's multiple range test) was used to compare differences in the data at a significance level of $P<0.05$.

3. Results

3.1. Identification of ERβ2 cDNA

RT-PCR was used to clone a fragment of ERβ2 cDNA using total RNA. A single PCR product of the expected size (1075 base pairs [bp]) was obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding ERβ2. The full-length ERβ2 cDNA contained 2004 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 667 amino acids (GenBank accession no. EU346949) (Fig. 1). Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that ERβ2 amino acids display high identity with those of other species. The amino acid sequence of black porgy ERβ2 was compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: 94% with gilthead seabream ERβ2 (gERb2, AJ580048), 75% with Mozambique tilapia ERβ2 (mERb2, EU140820) and 73% with killifish ERβb (kERbb, AY570923). As expected, the amino acid sequence identify of black porgy ERβ2 with other ERs was highest in the DNA-binding domain (DBD or C domain, residues 173–253, about 95%) and in the ligand-binding domain (LBD or E domain, residues 311–549, about 89%) (Fig. 1).

The bpERβ2 amino acid sequence was found to have 38.8 and 49.0%, similarity compared to bpERα and bpERβ1. The bpERβ2 shows a high percentage of conservation in the DBD (88–90%) and LBD (57–73%) when compared to bpERα and bpERβ1 (Fig. 2).

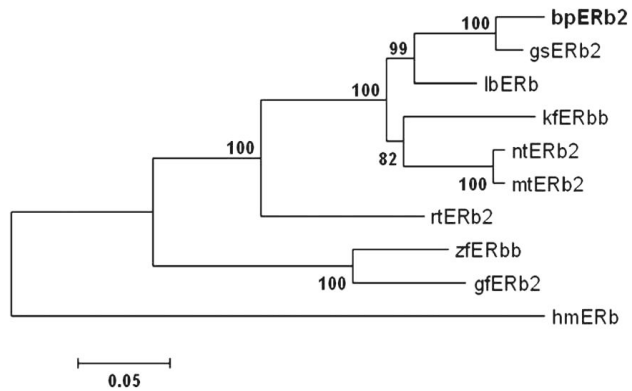

Fig. 3. Phylogenetic tree based on an amino acid alignment for ERβ2 in teleost fish. Bootstrap values (%) are indicated for 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: black porgy ERβ2 (bpERb2, this paper, EU346949), gilthead seabream ERβ2 (gsERb2, CAE30469), largemouth bass ERβ (lbERb, AAO39210), killifish ERβb (kfERbb, AAU44353), Nile tilapia ERβ2 (ntERb2, ABE73151), Mozambique tilapia ERβ2 (mtERb2, ABV55459), rainbow trout ERβ2 (rtERb2, ABB73309), zebrafish ERβb (zfERbb, AAK16741), goldfish ERβ2 (gfERb2, AAF35170) and human ERβ (hmERb, Q62986).

3.2. Phylogenetic analysis

The phylogenetic tree obtained by clustal analysis of the sequences described below was shown in Fig. 3. The phylogenetic analysis indicated that there are four groups of ERβ2 among the teleosts examined: group 1 (black porgy, gilthead seabream and largemouth bass), group 2 (killifish, Nile tilapia and Mozambique tilapia), group 3 (rainbow trout) and group 4 (zebrafish and goldfish). The ERβ2 is the most closely related to gilthead seabream ERβ2 (Fig. 3).

3.3. Quantification of the ERα, β1, and β2 mRNA expression

The expression changes of ERα, β1 and β2 mRNA by QPCR were shown in Fig. 4. The expression of ERα mRNA was highest in mature ovary, followed by mature testis, testicular portion of mostly testis (Fig. 4A). ERβ1 expression was higher in the mature testis and ovary than in the gonads at other maturity stages (Fig. 4B). In contrast to that, ERβ2 was highest in mature ovary, and significantly lower levels of ERβ2 expression were observed in the gonads of other maturity stages (Fig. 4C).

3.4. Histological analysis

The histological examination of the developmental stages of gonad was shown in Fig. 5. Testicular tissue was dominant in the mature testis with few primary oocytes (Fig. 5A). Also, the testicular portion of the gonad was degenerated (Fig. 5B) and the ovarian portion of the gonad was increased (Fig. 5C) during sex changing to female, and we observed oocytes including vitellogenic granule in the mature ovary (Fig. 5D).

4. Discussion

In this study, we cloned the full-length ERβ2 cDNA from the ovaries of mature female black porgy and investigated the changes in expression of ERα, ERβ1, and ERβ2 over time using QPCR. The ERβ2 cDNA from the black porgy contains a total of 2004 nucleotides that encode 667 amino acids in a single ORF. Using the BLAST algorithm (Blastp) from the National Center for Biotechnology Information, we found that the primary sequence of ERβ2 from the black porgy displayed significant identity with that from other species (94% with gilthead seabream ERβ2, 75% with Mozambique tilapia ERβ2, and 73% with killifish ERβb; Fig. 1).

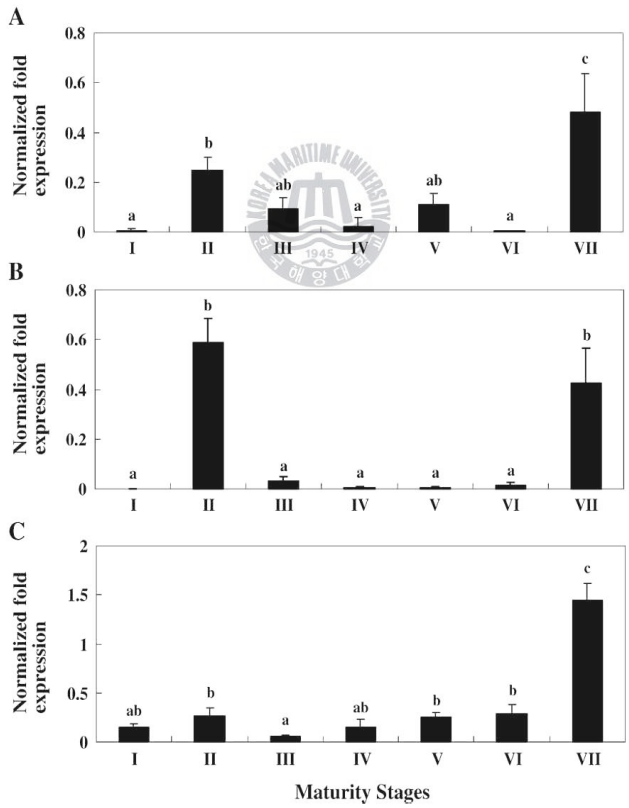

Fig. 4. Expression of ERα (A), ERβ1 (B) and ERβ2 (C) mRNA in the gonad of black porgy by quantitative real-time PCR. 3 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III: testicular portion of mostly testis, IV: ovarian portion of mostly testis, V: testicular portion of mostly ovary, VI: ovarian portion of mostly ovary, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

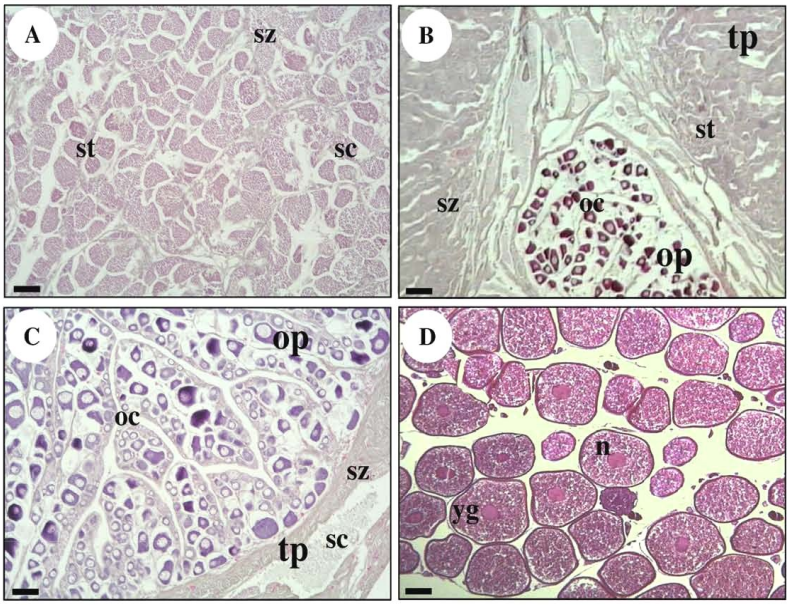

Fig. 5. Photomicrograph of gonad maturity stages during the sex change of black porgy. A: mature testis, B: mostly testicular gonad (III and IV in Fig. 4), C: mostly ovarian gonad (V and VI in Fig. 4), D: mature ovary, tp: testis part, st: spermatids, sz: spermatozoa, sc: spermatocytes, op: ovary part, oc: oocytes, n: nucleus, yg: yolk granules. Scale bar = 100 μm.

Black porgy ERβ2 has a similar domain structure to human, which was divided into six distinct (A–F) domains by Krust et al. (1986). Black porgy ERβ2 shows a high degree of conservation in its C (88–90%) and E (57–73%) domains with those in black porgy ERα and ERβ1 (Fig. 2), while the A/B domain (residues 1–172), which is similar in length with that in other fish, has two potential phosphorylation sites for mitogen-activated protein kinase (MAPK) ($PX_{1or2}SP$; residues 27–30 and 92–96; Fig. 1), suggesting that it may be phosphorylated in response to the MAPK pathway, a mechanism that has been shown to enhance the transcriptional activity of mammalian ERα and ERβ in a ligand-independent manner (Tremblay et al., 1997; Lannigan, 2003). Moreover, two zinc fingers (residues 179–200 and 215–234) are present in the DBD. The percent identity of ERβ2 from black porgy with that from other fish was highest in the DBD (domain C, residues 173–253, about 95%) and LBD (domain E, residues 311–549, about 89%). In addition, the activation function (AF)-2 domain (DLLLEMLD, located in the LBD at residues 537–544) (Kumar et al., 1987; Danielian et al., 1992; Pinto et al., 2006), which is involved in receptor dimerization, ligand-binding, and ligand-dependent transactivation, was well conserved in black porgy ERβ2 (Pinto et al., 2006) (Fig. 1).

The function of the F domain, which is similar in length to that in other species, is not well understood; however, it may interact with cofactors required for ER/Sp1 activity (Kim et al., 2003), which controls the magnitude of gene transcription (Montano et al., 1995) and inhibits dimerization of the receptor through an interaction with the E domain (Peters and Khan, 1999). Therefore, black porgy ERβ2 is similar to other members of the ERβ2 family in terms of its general features, and it contains all of the recognized consensus sequences.

Phylogenetic analysis of ERβ2 identified four subgroups (group 1: black porgy, gilthead seabream, and largemouth bass; group 2: killifish, Nile tilapia, and Mozambique tilapia; group 3: rainbow trout; and group 4: zebrafish and goldfish). The ERβ2 in black porgy was most closely related to that in gilthead seabream (Fig. 3). Notably, a third ER, called ERγ in the Atlantic croaker (Hawkins et al., 2000), was named ERβ2 in sea bass (Halm et al., 2004), goldfish (Ma et al., 2000), and gilthead seabream (Pinto et al., 2006). Based on our proposed nomenclature and phylogenetic analysis, we have designated the protein ERβ2.

Using QPCR, changes in the mRNA expression of ERα, ERβ1, and ERβ2 were investigated in the gonads of black porgy during the change in sex from male to female. A high level of ERα mRNA expression was observed in the mature testis and mature ovary, respectively; similarly, a high level of expression was observed in those gonads containing a large testicular portion during the sex change process. These results are in agreement with those from studies on the gilthead seabream (Socorro et al., 2000) and black porgy (He et al., 2003). In this study, ERα expression was higher in the mature ovary than in the testis, which contradicts the result of a previous study (He et al., 2003) in which higher expression was observed in the testis. These opposing results are presumed to be due to differences in the age and level of maturity of the black porgy used in each set of experiments. Although little published data are available on ERα expression in the gonads during sex change, ERα can be deduced from the results of this study to play an important role in the sexual development of black porgy because ERα transcription took place not only in the ovary of mature females, but also in the testis and testicular portion of the gonads of mature males during the sex change process. A high level of ERβ1 mRNA expression was observed in the mature testis and mature ovary, similar to the trend seen for ERα. Also, given that high levels of ERβ expression are important during sexual differentiation and development (Byers et al., 1997; Halm et al., 2004), high ERβ1 mRNA expression in mature testis of black porgy indicates that ERβ1 functions in the development and maturation of the gonads. Furthermore, we observed high levels of ERα and ERβ1 mRNA expression not only in mature ovary, but also in testis. This result corresponds to that of a previous study in goldfish (Choi and Habibi,

2003) and gilthead seabream (Pinto et al., 2006) showing high levels of receptor expression in the testis. This is an interesting result that underscores the potential functions of ERs during sexual differentiation and development as well as the novel function of estrogen in the male reproductive system (Couse and Korach, 1999; Halm et al., 2004).

In contrast to the above findings, ERβ2 was highly expressed only in mature ovary, with levels slowly increasing from testicular portion of mostly testis through to mature ovaries. This is in accordance with the results of Choi and Habibi (2003) and Halm et al. (2004), which showed greater ERβ2 mRNA expression in the ovaries than in the testis of mature goldfish and European sea bass respectively. Although ERβ1 is known to play an important role in the sexual differentiation and development of mammalian gonads (Byers et al., 1997), considering the high level of ERβ2 expression in the ovaries of mature female black porgy, ERβ2 appears to play an important role in the mature ovary.

Since both ERα and ERβ1 are expressed highly in mature testis and ovaries, we can speculate that they are involved in the maintenance and normal function of mature gonadal tissue. In contrast, ERβ2 expression is at its lowest at the onset of sex change, and slowly increases through the process, reaching a maximum in mature ovaries. This indicates that 1) ERβ2 may be directly involved in sex change and 2) it is likely to contribute along with ERα and ERβ1 to the maintenance and function of mature ovarian processes.

Histological analysis of each stage of the sex change process during the spawning season revealed the presence of sperm in the gonads, indicating the existence of functionally mature gonads, and oocytes containing numerous vitellogenic granules, which are indicative of impending ovulation, were observed in the ovaries of the mature females. Moreover, as the switch to ovaries occurred in the bisexual gonads, degeneration of the testicular tissues was observed as were oocytes with chromatin in the nucleolus and peri-nucleolus stages. Oocytes with secondary yolk (i.e., globule stage), which is characteristic of mature oocytes just before ovulation (Wu et al., 2005), were also observed in the mature ovaries (Fig. 5). We found mature oocytes in the ovaries of the mature female at the same time as we detected high levels of ER mRNA expression. This is in accordance with the results of a previous study (Filby and Tyler, 2005) showing a higher level of ER mRNA expression in the testis and ovary than in the immature gonad of male and female fathead minnows (*Pimephales promelas*).

In summary, full-length ERβ2 cDNA was separated from the ovary of mature female black porgy, and using QPCR, the mRNA expression of three ER subtypes (ERα, ERβ1, and ERβ2) in the gonad was compared at each stage of the sex change process. Our results indicate a high level of expression in the mature testis and ovary, from which we deduce that ERs play an important role in gonadal maintenance and function while ERα and ERβ1 are down-regulated during sex change, implicating the role of ERβ2 as a potential modulator of sex change since its transcript slowly increases. Additional studies will be necessary to determine the roles of ERs in gonadal development and maturation. Furthermore, the results of this study can be used to elucidate the endocrinological mechanism of sex change in black porgy.

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III. Experiment 2

Molecular Characterization and Expression of Three GnRH forms mRNA During Gonad Sex-Change Process, and Effect of GnRHa on GTH subunits mRNA in the Protandrous Black Porgy, *Acanthopagrus schlegeli*

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Molecular characterization and expression of three GnRH forms mRNA during gonad sex-change process, and effect of GnRHa on GTH subunits mRNA in the protandrous black porgy (*Acanthopagrus schlegeli*)

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in control of reproduction and gonadal maturation in teleost fish. To investigate the action GnRH in black porgy (*Acanthopagrus schlegeli*), we examined the mRNA expression of GTH subunits (GTHα, FSHβ, and LHβ) in the pituitary as well as plasma estradiol-17β (E_2) level following treatment with a GnRH analog (GnRHa) in immature fish. The expression levels of GTH subunits mRNA and plasma E_2 level were increased after GnRHa injection. We were also able to identify three GnRH forms: salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II) by cDNA cloning in the ovary of the black porgy. Black porgy gonadal development is divided into seven stages, involving sex change from male to female (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary). In the present study, we investigated the expression pattern of three GnRH molecular forms in the black porgy gonads at different stages of gonadal development by quantitative polymerase chain reaction (QPCR). The mRNA expressions of sGnRH, sbGnRH and cGnRH-II were found to be higher in mature testis and ovary, compared to gonads at different stages of maturity. The findings support the hypothesis that the three forms of GnRH play important roles in the regulation of hypothalamic–pituitary–gonadal axis, and are likely involved also in gonadal development and sex change in black porgy.

1. Introduction

The regulation of reproduction in teleost fish is a complex process involving the interaction of a number of factors including gonadotropin-releasing hormone (GnRH), gonadotropins, gonadal hormones, and other neurohormones. GnRH is released by the hypothalamus and stimulates the synthesis and release of hypophysial gonadotropin hormones (GTHs): follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The GTHs have two subunits: GTHα, which is common to FSH, LH and thyroid stimulating hormone, and a β subunit which is specific to either FSH or LH. Pituitary LH stimulates the synthesis and secretion of steroid hormones from the gonads, and FSH regulates both vitellogenesis and spermatogenesis, thereby regulating ovarian and testicular function in teleosts (for review see: Ando and Urano, 2005). Thus, gonadal maturation is primarily regulated by the brain–pituitary–gonadal axis, and GnRH plays a central role in the regulation of gonadal maturation and reproduction in fish and other species.

To date, 15 GnRH isoforms have been isolated from vertebrates, comprising a family of highly conserved, decapeptide neurohormones responsible for the control and coordination of reproduction in all vertebrates (Kavanough et al., 2008). Typically, several forms of GnRH are co-expressed in the brain of vertebrates. The majority of vertebrates studied to date express chicken GnRH-II (cGnRH-II), which appears to be largely ubiquitous. Similarly, sea bream, a protandrous hermaphroditic fish, express three forms of GnRH: salmon GnRH (sGnRH), cGnRH-II, and seabream GnRH (sbGnRH) in the brain and gonads (Gothilf et al., 1995; Nabissi et al., 2000).

All GnRH forms identified have a primary gene structure, which is highly conserved. GnRH is encoded as a prepro-hormone, consisting of a "single peptide", directly followed by the decapepdide, and by a "GnRH-associated peptide" (GAP) (Guilgur et al., 2006). Interestingly, extra-hypothalamic GnRH has been reported in the ovaries and testes of various species, including the seabream (Andreu-Vieyra et al., 2005; Nabissi et al., 2000; Soverchia et al., 2007). It has been suggested that GnRH is involved in the regulation of gonadal function as an autocrine or paracrine regulator (Andreu-Vieyra et al., 2005; Leung and Steele, 1992), and direct actions of GnRH on the resumption of oocyte meiosis and effects on steroidogenesis have also been

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reported in goldfish (Habibi et al., 1988, 1989). Additionally, gonadal GnRH appears to regulate testicular and ovarian apoptosis in goldfish and gilthead seabream, which might be an important factor in follicular astresia, control of spermatogenesis, and early sex differentiation in fish (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra et al., 2005; Soverchia et al., 2007). Nabissi et al. (2000) identified GnRH transcripts in the gilthead seabream during the sex-change process, suggesting that GnRHs may be involved in the paracrine/autocrine regulation of seabream sex change from male to female. Despite these recent insights, the molecular mechanism of gonadal sex change in protandrous hermaphroditic fish remains poorly understood.

Black porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae), are marine protandrous hermaphrodites that are widely distributed and are of particular interest for commercial aquaculture in parts of Asia including Korea. These fish are functional males for their first 2 years of life, but approximately 70% of black porgy change into females during the third spawning season in their natural environment.

The objective of this study was two fold: (1) To test the hypothesis that GnRH regulates induction of pituitary LH and FSH, and the subsequent increase in circulating estradiol-17β (E_2) levels in immature black porgy (1-year-old). (2) To investigate the expression of GnRH transcripts during the sex-change process, with associated changes in pituitary GTHα, LHβ and FSHβ, and circulating E_2 levels.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature fish (51.0 ± 2.3 g, 1-year-old), mature male (220 ± 14.2 g, 2-year-old), sex changing fish (489.2 ± 11.5 g, 3-year-old) and female black porgy (948.5 ± 51.6 g, 4-year-old). The fish were captured in spawning period (May, water temperature: 20 °C) and sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were anesthetized in tricaine methane sulfonate (MS-222, Sigma–Aldrich, St. Louis, MO, USA), prior to blood collection. Blood was collected from the caudal vasculature using a 3 ml syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000g, 5 min) and stored at −80 °C until RIA analysis. Gonad and pituitary samples from black porgy at each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and mature ovary) were removed, immediately frozen in liquid nitrogen and stored at −80 °C until the total RNA was extracted for analysis.

2.2. GnRHa treatment

To establish that GnRH was active in black porgy, we first injected immature fish with GnRHa and measured pituitary expression of GTHα, LHβ and FSHβ, and associated changes in circulating E_2. GnRHa (des Gly^{10}-[D-Ala^6] LHRH ethylamide, Sigma) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the fish were given an injection of GnRHa (0.2 μg/g, body weight, BW) at volume of 1 μl/g BW. After injection, pituitary and blood were sampled from three fish at each of the following time periods: 0, 6, 12, 24, and 48 h. Water temperature was maintained 20 ± 1 °C during the injection periods. All fish survived the experimental periods.

2.3. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from gonad and pituitary of black porgy at the each gonad maturity stages during sex-change process (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary) and pituitary (GnRHa treatment fish), using the Trizol method, according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. About 2.5 μg of total RNA was reverse transcribed in a total volume of 20 μl, using an oligo-$d(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.4. Identification of sGnRH, sbGnRH, and cGnRH-II cDNA

The primers used for three GnRHs amplification were designed using highly conserved regions of other teleost fish; sGnRH forward primer (5′-GCA GAG TGA CGG TGC AGG TG-3′), sGnRH reverse primer (5′-CTT CCG GTC GAA AGG ACT GG-3′), sbGnRH forward primer (5′-CCA CAG ACT TCA AAC CTC TGG-3′), sbGnRH reverse primer (5′-GTA CGT TCT GTG TCC GTT GT-3′), cGnRH-II forward primer (5′-CTC GGC TGG TTT TGC TGC TC-3′), and cGnRH-II reverse primer (5′-CTC TTC TGG AGC TCT CTT GC-3′). Total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). PCR amplification was performed using a 2× Taq Premix I (Solgent, Daeseon, Korea) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 40 s, and extension at 72 °C for 60 s; followed by 7 min at 72 °C for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Sungnam, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Seoul, Korea) and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, the sGnRH, sbGnRH, and cGnRH-II cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR, total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo-$(dT)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene).

Gene specific primers were selected from the PCR product obtained by RT-PCR in the present study. For the 3′ RACE, the 50 μl of PCR mixture contained 5 μl of 3′ RACE cDNA, 1 μl of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 μM 3′ RACE sGnRH-specific primer (5′-GAG CTG GAG GCA ACC ATC AGA ATG ATG G-3′), 1 μl of 10 μM 3′ RACE sbGnRH-specific primer (5′-GCT GTC AGC ACT GGT CCT ATG GAC TG-3′), 1 μl of 10 μM 3′ RACE cGnRH-II-specific primer (5′-CAA GAG GGA GCT GGA CTC TTT TGG CAC-3′), and 25 μl of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μl of PCR mixture contained 5 μl of 5′ RACE cDNA, 1 μl of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 μM 5′ RACE sGnRH-specific primer (5′-CCA TCA TTC TGA TGG TTG CCT CCA GCT C-3′), 1 μl of 10 μM 5′ RACE

sbGnRH-specific primer (5′-GTA CGT TCT GTG TCC GTT GT-3′), 1 μl of 10 μM 5′ RACE cGnRH-II-specific primer (5′-GTG CCA AAA GAG TCC AGC TCC CTC TTG-3′), and 25 μl of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis in a 1% agarose gel containing ethidium bromide (Biosesang). The transformation was conducted as the same methods mentioned above.

2.6. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length sGnRH, sbGnRH, and cGnRH-II cDNA from various fishes. Amino acid sequences were aligned using the BioEdit Software (Hall, 1999). Sequences used for comparison and their GenBank accession numbers are as follows: sGnRH [black porgy sGnRH (EU117212, this paper), gilthead seabream sGnRH (AF046799), red seabream sGnRH (D26108), flathead mullet sGnRH (AY373449), Nile tilapia sGnRH (AB104863), spotted weakfish sGnRH (AAV74403), bluefin tuna GnRH3 (ABX10868), Atlantic croaker sGnRH (AAQ16503), European sea bass sGnRH (AF224280), cobia sGnRH (AY677173)], sbGnRH [black porgy sbGnRH (EU099997, this paper), red seabream sbGnRH (D86582), gilthead seabream sbGnRH (AF046801), Nile tilapia sbGnRH (AB104861), flathead mullet sbGnRH (AY373450), cobia sbGnRH (AY677175), bluefin tuna GnRH1 (EU239500), barfin flounder sbGnRH (DQ074693), European sea bass sbGnRH (AF224279)], cGnRH-II [black porgy cGnRH-II (EU099996, this paper), gilthead seabream cGnRH-II (U30325), rainbow trout cGnRH-II (AF125973), lake whitefish cGnRH-II (AY245102), Nile tilapia cGnRH-II (AB104862), Atlantic croaker cGnRH-II (AY324669), European sea bass cGnRH-II (AF224281), striped sea bass cGnRH-II (AF056313), bluefin tuna GnRH2 (EU239502), cobia cGnRH-II (AY677174), spotted weakfish cGnRH-II (AY796309), bastard halibut cGnRH-II (DQ008580), barfin flounder cGnRH-II (AB066359), and flathead mullet cGnRH-II (AY373451)], and chicken GnRH1 (X69491), and human GnRH1 (NP_000816) were used as the outgroup. The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.7. Quantitative polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative expression of three GnRH isoforms (sGnRH, sbGnRH, and cGnRH-II) and GTH subunits (GTHα, FSHβ, and LHβ) mRNA using total RNA extracted from the gonads and pituitary of black porgy, respectively. Primers for QPCR were shown in Table 1. QPCR amplification was conducted similar to previous work (Nelson et al., 2007), using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and with the following conditions: 0.5 μl of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr green, and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μl. QPCR was carried out as follows: one cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 96.3%, sGnRH = 94.2%, sbGnRH = 95.0%, cGnRH-II = 96.4%, GTHα = 93.8%, FSHβ = 91.4%, and LHβ = 92.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

Table 1
Primers used for amplification of QPCR

Genes	DNA sequences	Accession no.
sGnRH-F	5′-GCG AGC AGC AGA GTG ACG-3′	EU117212
sGnRH-R	5′-TTC TCT TCC CAC CTG GTA GCC-3′	
sbGnRH-F	5′-GGT GGT GAT GAT GAT GAT GAT GTC-3′	EU099997
sbGnRH-R	5′-AAT GTT GCC CAG CGT GTC C-3′	
cGnRH-II-F	5′-GCT CGG GCT GCT CCT ATG-3′	EU099996
cGnRH-II-R	5′-CTC CTC TGA AAT CTC TGA TGT GC-3′	
GTHα-F	5′-AAG ACG ATG ACG ATC CCG AAG-3′	EF605275
GTHα-R	5′-GTG TGG TTC CTC ACC CTT ATG C-3′	
FSHβ-F	5′-TGC CAT CCA ACC AAC ATC AGC-3′	AY921613
FSHβ-R	5′-ATC CTC GTG GTA GCA CTG TCC-3′	
LHβ-F	5′-ACC AAG GAC CCA GTG ATG AAG AC-3′	EU605276
LHβ-R	5′-GGG CGG ACA GTC AGG AAG C-3′	
β-actin-F	5′-GGA CCT GTA TGC CAA CAC TG-3′	AY491380
β-actin-R	5′-TGA TCT CCT TCT GCA TCC TG-3′	

2.8. Plasma parameters analysis

Plasma estradiol-17β (E_2) levels were analyzed by radioimmunoassay (RIA) using E_2 RIA kit (DIASORIN, Antony, France).

2.9. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Newman Keul's multiple range test) was used to compare differences in the data at a significance level of $P < 0.05$.

3. Results

3.1. Effects of GnRHa on pituitary mRNA expression of GTHα, FSHβ, and LHβ and circulating E_2 levels

The time-related changes in pituitary expression of GTHα, FSHβ, and LHβ mRNA after treatment with GnRHa are shown in Fig. 1. The GTHα mRNA was significantly increased at all time points with a maximum at 24 h (approximately 10.9-fold higher than that of the control) and then decreased at 48 h post-GnRHa injection. The FSHβ mRNA increased to a maximum at 12 h (approximately 6.4-fold higher than that of the control) and then decreased at the end of the experiment. On the other hand, the LHβ mRNA was increased to approximately 2.0-fold higher than control fish by 6 h and remained high through the remainder of the experiment (up to 48 h).

The associated changes in plasma E_2 caused by GnRHa treatment are shown in Fig. 2. As expected, the plasma E_2 level increased from 9.0 ± 0.7 pg/ml at the start of the experiment to 21.1 ± 5.4 pg/ml after 24 h, and reached a maximum level of 44.7 ± 3.7 pg/ml by the end of the experiment (after 48 h).

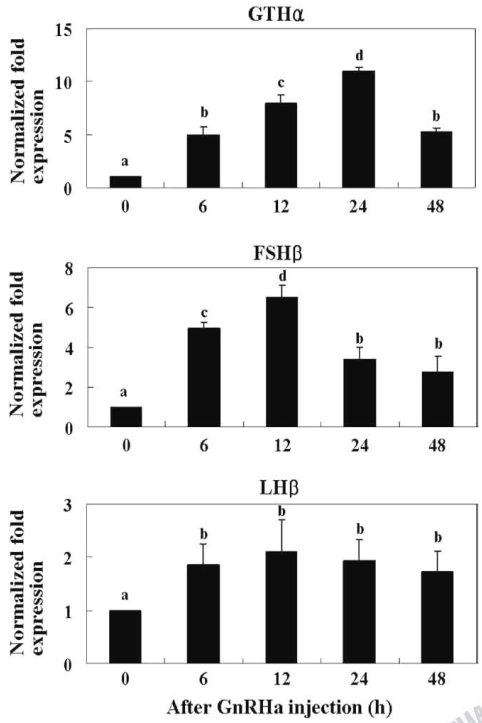

Fig. 1. Expression of GTHα, FSHβ and LHβ mRNA in pituitary of black porgy using quantitative PCR by GnRHa injection. About 2.5 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

3.2. Identification of three GnRH forms

Primers for GnRH were designed based on sequence information available in seabream, since as in black porgy, this species is a member of the family Sparidae. RT-PCR was used to clone fragments of sGnRH, sbGnRH, and cGnRH-II cDNA using total RNA. Single PCR product of the expected sizes (sGnRH; 239 base pairs [bp], sbGnRH; 276 bp, and cGnRH-II; 241 bp) were obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding three GnRH forms.

The full-length sGnRH cDNA contained 273 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 90 amino acids (GenBank Accession No. EU117212), the full-length sbGnRH cDNA consists of 291 nucleotides, including an ORF that was predicted to encode a protein of 96 amino acids (EU099997), and the full-length cGnRH-II cDNA contained 258 nucleotides, including an ORF that was predicted to encode a protein of 85 amino acids (EU099996).

Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that GnRHs amino acids display high identity with those of other species. The amino acid sequences of three GnRH forms were compared to those deduced from the cDNA of other teleost species (Fig. 3). The amino acid similarities between the prepro-hormones were as follows: sGnRH; 100% with gilthead seabream sGnRH, 98% with red seabream sGnRH, 95% with bluefin tuna GnRH3 and 90% with spotted weakfish sGnRH, sbGnRH; 87% with red seabream sbGnRH, 74% with bluefin tuna

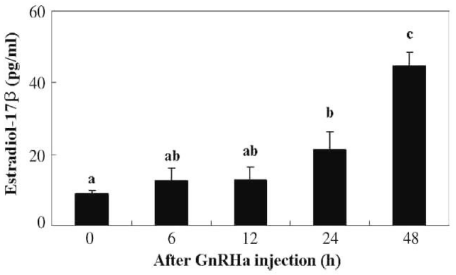

Fig. 2. The plasma estradiol-17β (E_2) levels by GnRHa injection in black porgy. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD ($n=3$).

GnRH1, 65% with flathead mullet sbGnRH, and 63% with European sea bass sbGnRH, cGnRH-II; 98% with gilthead seabream cGnRH-II, 96% with bluefin tuna GnRH2, 96% with spotted weakfish cGnRH-II, and 95% with Nile tilapia cGnRH-II.

The three GnRH cDNAs found in black porgy all consisted of the characteristic signal peptides (sGnRH; 1–23 residues, sbGnRH; 1–26 residues, and cGnRH-II; 1–26 residues), specific GnRH amino acids (sGnRH; 24–33 residues, sbGnRH; 27–36 residues, and cGnRH-II; 24–33 residues), enzymatic processing site (Gly-Lys-Arg [G-K-R], sGnRH; 34–36 residues, sbGnRH; 37–39 residues, and cGnRH-II; 34–36 residues), and GnRH-associated peptides (GAP) (sGnRH; 37–90 residues, sbGnRH; 40–96 residues, and cGnRH-II; 37–85 residues).

3.3. Phylogenetic analysis

The phylogenetic tree obtained by clustal analysis of the sequences described below is shown in Fig. 4. Phylogenetic analysis indicated a strong relationship among the same GnRH forms. Therefore, based on this data, we designated the names of the three black porgy GnRH forms. It is also important to note that the black porgy GnRHs were the most closely related to fellow members of the Perciformes order, the gilthead seabream (sGnRH and cGnRH-II) and red seabream (sbGnRH).

3.4. Expression of sGnRH, sbGnRH, and cGnRH-II mRNA throughout the sex-change process

Since GnRH has been implicated in the sex-change process in sea bream (Nabissi et al., 2000; Soverchia et al., 2007), we investigated the gonadal expression of the newly identified GnRH forms throughout sex change (Fig. 5). QPCR analysis revealed that the expression patterns between the three GnRH forms were very similar throughout the life cycle of black porgy. Specifically, all three identified GnRH forms were higher in mature testis and mature ovary than in either immature gonads or sex-switching gonads. sbGnRH and cGnRH-II transcripts were significantly higher in mature ovaries than in testis, while sGnRH transcripts were similar between mature testis and mature ovaries.

3.5. Expression of GTHα, FSHβ and LHβ mRNA in pituitary, and circulating E_2 levels throughout the sex-change process

In an effort to create a comprehensive framework of physiological changes during the sex change process, we investigated associated changes in pituitary GTHα, FSHβ, and LHβ mRNA expression, as well as plasma E_2 levels. GTHα mRNA was found to be the lowest in immature fish, with higher expression noted in mature male testis, sex-switching fish and fish with mature ovaries (Fig. 6). Pituitary FSHβ expression was the highest in mature males with

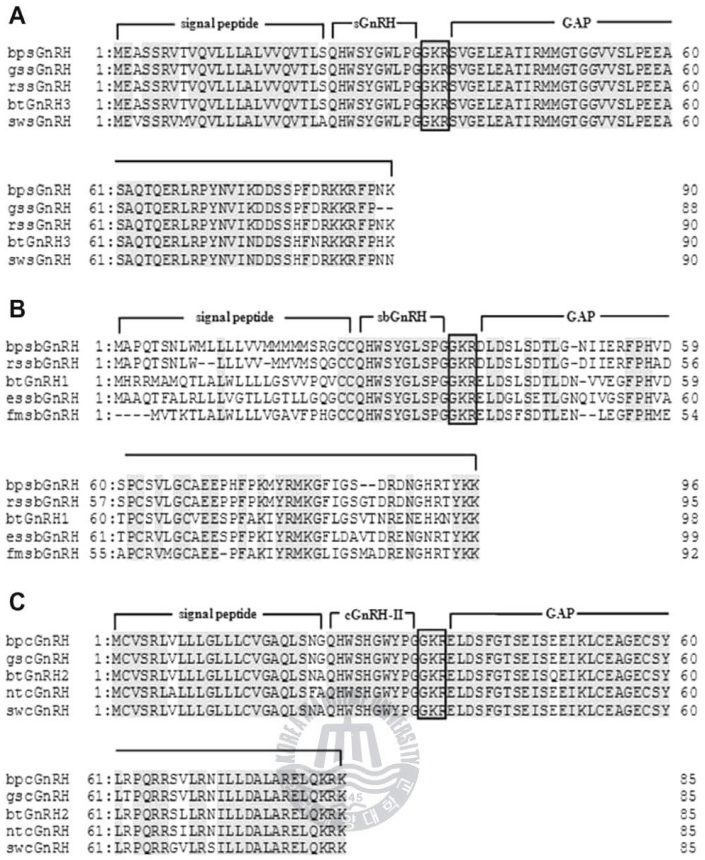

Fig. 3. Comparison of the amino acid sequence of sGnRH (A), sbGnRH (B), and cGnRH-II (C). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The amino acid sequences of black porgy sGnRH (bpsGnRH, this paper, EU117212), gilthead seabream sGnRH (gssGnRH, AF046799), red seabream sGnRH (rssGnRH, D26108), bluefin tuna GnRH3 (btGnRH3, ABX10868), spotted weakfish sGnRH (swsGnRH, AAV74403), black porgy sbGnRH (bpsbGnRH, this paper, EU099997), red seabream sbGnRH (rssbGnRH, D86582), bluefin tuna GnRH1 (btGnRH1, EU239500), European sea bass sbGnRH (essbGnRH, AF224279), flathead mullet sbGnRH (fmsbGnRH, AY373450), black porgy cGnRH-II (bpcGnRH, this paper, EU099996), gilthead seabream cGnRH-II (gscGnRH, U30325), bluefin tuna GnRH2 (btGnRH2, EU239502), Nile tilapia cGnRH-II (ntcGnRH, AB104862), and spotted weakfish cGnRH-II (swcGnRH, AY796309) optimally aligned to match identical residues, indicated by the shaded box. The enzymatic processing site (GKR) is boxed.

intermediate levels in immature fish and fish with mostly testicular gonads, and lower levels as fish switch to having mostly ovarian and completely ovarian gonads (Fig. 6). LHβ mRNA started off low in immature fish, increased by ~3-fold in mature testes, was reduced again as the gonads started to switch but then increased again as the gonads increased on ovarian character, with the highest pituitary expression in mature females (Fig. 6).

Plasma E_2 levels were shown Fig. 7 and highest in mature female. It was found to be low in immature male fish (9.46 ± 1.6 pg/ml) increased slightly in mature males (10.45 ± 1.48 pg/ml), decreased again at the beginning of the sex change, rising as more ovarian content was observed (switching mostly testis: 5.0 ± 0.4 pg/ml, switching mostly ovary gonad: 6.0 ± 1.4 pg/ml) and dramatically increased to a maximum of 705.6 ± 70 pg/ml in mature females (Fig. 7).

4. Discussion

It is well established that in vertebrates, including teleosts, hypothalamic GnRH plays a pivotal role in the regulation of steroidogenesis and ovulation by mediating the synthesis and release of the GTHs. Specifically GnRH agonist treatment was found to increase GTHα and LHβ mRNA expression in striped sea bass (Hassin et al., 1998), sockeye salmon (Ando and Urano, 2005) and coho salmon (Dickey and Swanson, 2000). The two native forms found in goldfish sGnRH and cGnRH-II increased GTHα, LHβ and FSHβ above control levels *in vivo* and *in vitro* (Huggard-Nelson et al., 2002; Klausen et al., 2002). In the related red seabream, GnRHa increased the mRNA expression levels for the GTH subunits as well as plasma E_2 levels (Kumakura et al., 2004). Here, we found that the mRNA expression of all three GTH subunits increased in the pituitary. Additionally, associated increases in plasma E_2 levels were also observed after GnRHa treatment in immature black porgy. These data suggested that GnRHa directly increased the expression levels of GTH subunits mRNA in the black porgy pituitary.

Full-length GnRH cDNA was isolated in the mature ovaries of black porgy and the amino acid sequences for the three GnRH forms are highly similar to those of other fish species. Many studies have shown the same general organization for GnRHs, with

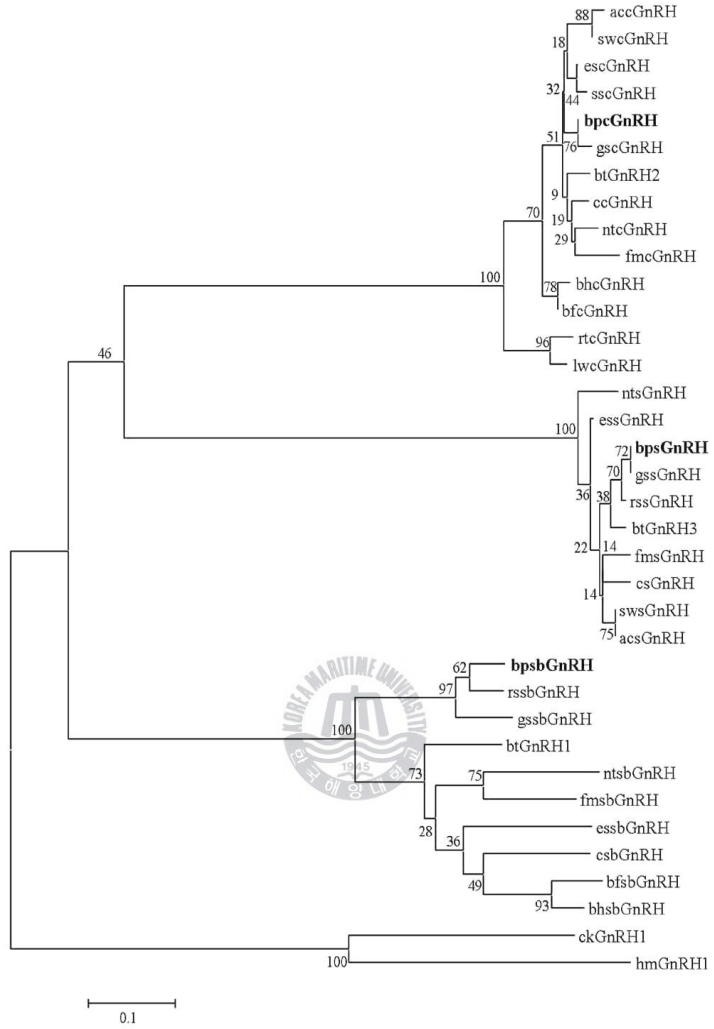

Fig. 4. Phylogenetic tree based on an amino acid alignment for sGnRH, sbGnRH, and cGnRH-II in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: black porgy sGnRH (bpsGnRH, this paper, EU117212), gilthead seabream sGnRH (gssGnRH, D26108), flathead mullet sGnRH (fmsGnRH, AY373449), Nile tilapia sGnRH (ntsGnRH, AB104863), spotted weakfish sGnRH (swsGnRH, AAV74403), bluefin tuna GnRH3 (btGnRH3, ABX10868), Atlantic croaker sGnRH (acsGnRH, AAQ16503), European sea bass sGnRH (essGnRH, AF224280), cobia sGnRH (csGnRH, AY677173), black porgy sbGnRH (bpsbGnRH, this paper, EU099997), red seabream sbGnRH (rssbGnRH, D86582), gilthead seabream sbGnRH (gssbGnRH, AF046801), Nile tilapia sbGnRH (ntsbGnRH, AB104861), flathead mullet sbGnRH (fmsbGnRH, AY373450), cobia sbGnRH (csbGnRH, AY677175), bluefin tuna GnRH1 (btGnRH1, EU239500), barfin flounder sbGnRH (bfsbGnRH, AB066360), bastard halibut sbGnRH (bhsbGnRH, DQ074693), European sea bass sbGnRH (essbGnRH, AF224279), black porgy cGnRH-II (bpcGnRH, this paper, EU099996), gilthead seabream cGnRH-II (gscGnRH, U30325), rainbow trout cGnRH-II (rtcGnRH, AF125973), lake whitefish cGnRH-II (lwcGnRH, AY245102), Nile tilapia cGnRH-II (ntcGnRH, AB104862), Atlantic croaker cGnRH-II (accGnRH, AY324669), European sea bass cGnRH-II (escGnRH, AF056313), bluefin tuna GnRH2 (btGnRH2, EU239502), cobia cGnRH-II (ccGnRH, AY677174), spotted weakfish cGnRH-II (swcGnRH, AY796309), bastard halibut cGnRH-II (bhcGnRH, DQ008580), barfin flounder cGnRH-II (bfcGnRH, AB066359), flathead mullet cGnRH-II (fmcGnRH, AY373451), chicken GnRH1 (X69491), and human GnRH1 (NP_000816).

regions for the signal peptide, specific GnRH amino acids, an enzymatic processing site, and GAP present in all species (Guilgur et al., 2006). Comparison of amino acid sequences of three black porgy GnRH forms with other fish species revealed that the black porgy specific GnRH amino acids and enzymatic processing site were similar to other fish (Amano et al., 1997; Guilgur et al., 2007) (Fig. 3). Therefore, based on the specific GnRH amino acid sequences, the present study demonstrates the presence of three forms of GnRH, including sGnRH, sbGnRH, and cGnRH-II in black porgy.

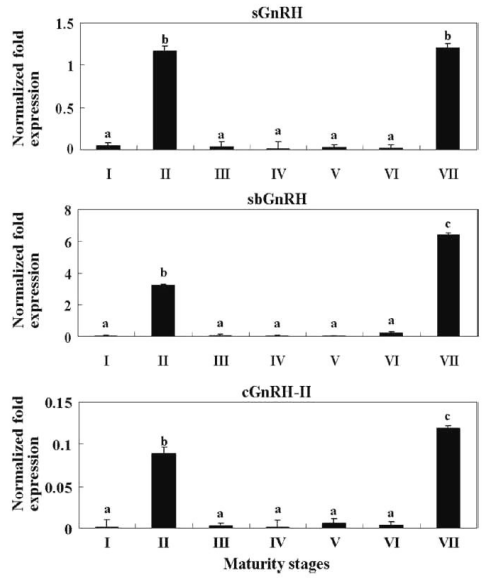

Fig. 5. Expression of sGnRH, sbGnRH, and cGnRH-II mRNA in gonad of black porgy by quantitative real-time PCR. About 2.5 μg of total RNA prepared from gonad was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex-change process from male to female (I, immature testis; II, mature testis; III, testicular portion of mostly testis; IV, ovarian portion of mostly testis; V, testicular portion of mostly ovary; VI, ovarian portion of mostly ovary; VII, mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

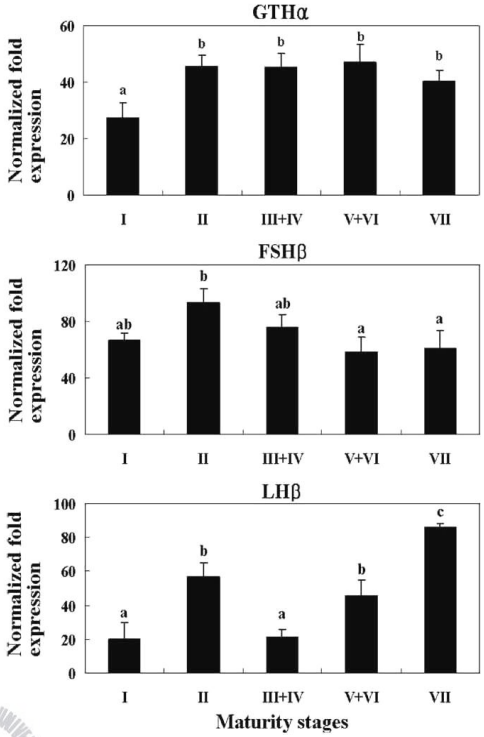

Fig. 6. Expression of GTHα, FSHβ, and LHβ mRNA in pituitary of black porgy by quantitative real-time PCR. About 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into five stages during the sex-change process from male to female (I, immature testis; II, mature testis; III+IV, mostly testicular gonad; V+VI, mostly ovarian gonad; VII, mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

Phylogenetic analyses suggest that the three GnRH forms have likely derived from the same gene and that they are most closely related to the gilthead seabream and the red seabream of the Sparidae family. We also demonstrate that each of the three GnRHs was well conserved among other fish species (Fig. 4).

A number of studies have shown the expression of GnRH mRNA in the gonads of vertebrates including teleost fish [for example: gilthead seabream; (Nabissi et al., 1997, 2000), rat (Goubau et al., 1992), rainbow trout (Uzbekova et al., 2001), and the goldfish (Andreu-Vieyra et al., 2005; Pati and Habibi, 1998)]. However, few studies have examined the role of GnRHs in sex differentiation and sex change in fish. Specifically, Andreu-Vieyra et al. (2005) demonstrated that GnRH may be an important mediator of apoptosis and subsequent regression of the testis in the goldfish, a seasonal spawner. In the hermaphrodite the gilthead seabream, Soverchia et al. (2007) reported higher levels of GnRH mRNA in nascent ovaries than in mature regressing testes and the testicular portion of the switching gonad. Therefore, in order to better characterize the potential roles of GnRH in the black porgy sex-change process, we performed QPCR to examine the expression patterns of gonadal GnRH mRNA expression during the sex-change process. The mRNA transcript levels of all three forms of GnRH were found to be high in mature testes and ovaries. However, in comparison, immature gonads and gonads throughout the switching process had very low GnRH mRNA levels. These results are in accordance with previous evidence that GnRH mRNA was reduced in switching testis compared to mature testis, and generally highest in the mature ovary of the gilthead seabream (Soverchia et al., 2007). Our results for black porgy, demonstrate a significant decrease in GnRH transcript throughout the sex-change process. Therefore, our evidence suggests that the three GnRH forms may play critical roles in the sex-change process of this group of sequential hermaphrodites. The high levels in mature testis may help maintain the correct ratio of Sertoli cells and gametes through the stimulation of apoptosis in the testes (Lee et al., 1997, 1999; Soverchia et al., 2007).

Since the sex change likely involves several endocrine factors on a multi-level scale, we also examined the pattern of mRNA expression for the pituitary GTH subunits throughout the sex change. FSHβ mRNA expression was high in the pituitary of mature males, dropping slightly at the onset of the sex change and having significantly lower levels as the gonads became mostly ovarian. Similarly, LHβ mRNA expression was high in the pituitary of mature males and dropped significantly after the sex change was initiated. However, it then increased as the gonads became more ovarian, with the highest values in mature females. The common α subunit (GTHα) was low in immature fish, but then increased and remained high throughout the adult life cycle. Therefore, it is likely that FSH and LH also have important roles in the sex-change process. Indeed the profile for plasma LH in sex-switching black porgy is distinct from non-switching males (Lee et al., 2001). Treatment with exogenous E_2 has been shown to induce sex change (for review see: Lee et al., 2001), and GnRHa treatment has been shown to regulate a gonadal estrogen receptor (Choi et al., 2007), impli-

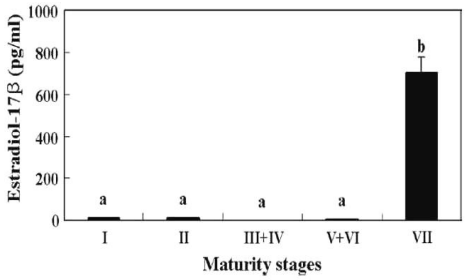

Fig. 7. The plasma estradiol-17β (E_2) levels during sex change of black porgy. (I, immature testis; II, mature testis; III+IV, mostly testicular gonad; V+VI, mostly ovarian gonad; VII, mature ovary). Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means±SD ($n=3$).

cating E_2 as a potential mediator of sex change. However, we find that plasma E_2 slowly increases as the gonads switch, with the only significant increase in concentration found in mature ovaries. This result is in accordance with previous studies of protandrous sea bass (Guiguen et al., 1993) and anemone fish (Godwin and Thomas, 1993), both of which reported that plasma E_2 levels were highest in mature females. This would correspond to more ovarian tissue available to synthesize E_2. Therefore, while exogenous E_2 can induce sex change, endogenous E_2 is likely the result of increased ovarian tissue, not the initial signal for sex change.

In summary, GnRHa was found to stimulate the expression of the pituitary gonadotropins and increase plasma E_2. Subsequently, three forms of GnRH (sGnRH, sbGnRH, and cGnRH-II) cDNA were isolated from the ovaries of mature female black porgy. Using QPCR, the mRNA expression of the three GnRH forms in the gonad and GTHs in the pituitary were compared at each stage of the sex-change process. Our results indicate a high level of GnRH expression in the mature testes and ovaries, from which we deduce that GnRHs play an important role in gonadal development and maturation. Additional studies will be necessary to determine the precise roles of the gonadal GnRHs and pituitary GTHs in gonadal development, maturation, and sex change. The results of this study provide a framework for future work investigating sex change as a multi-endocrine level mediated process in the black porgy and other sequential hermaphrodites.

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IV. Experiment 3

Molecular Characterization of Gonadotropin Subunits and Gonadotropin Receptors in Black Porgy, *Acanthopagrus schlegeli*: Effects of Estradiol-17β on mRNA Expression Profiles

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Molecular characterization of gonadotropin subunits and gonadotropin receptors in black porgy, *Acanthopagrus schlegeli*: Effects of estradiol-17β on mRNA expression profiles

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ABSTRACT

The cDNAs of three gonadotropin (GTH) subunits (GTHα, FSHβ, and LHβ) and two GTH receptors (FSHR and LHR) from pituitary and gonads of black porgy were cloned. The nucleotide sequences of the GTHα, FSHβ, and LHβ cDNA were 354, 363, and 414 base pairs (bps) in length with open reading frames (ORF) encoding peptides of 117, 120, and 137 amino acids, respectively. The FSHR and LHR cDNA was 2118 and 2076 bps in length with ORFs encoding peptides of 705 and 691 amino acids, respectively. To study the mechanism of the estradiol-17β (E_2) action, we examined the expression pattern of GTH subunit mRNAs in pituitary and GTH-receptor mRNAs in gonads, and the changes of plasma E_2 level when E_2 treatment was applied to immature black porgy. E_2 treatment increased mRNA expression levels of the genes and plasma E_2 levels, indicating that E_2 stimulated the increases in GTH subunit and GTH-receptor mRNAs. These data indicate that E_2 plays an important regulatory role in the brain–pituitary–gonad axis of immature black porgy. We provide the molecular characterization and expression of the GTH subunits and GTH receptors during sex change in the protandrous black porgy.

1. Introduction

Gonadotropin-releasing hormone (GnRH) from the hypothalamus induces the synthesis and release of the hypophysial gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which stimulate the secretion of the gonadal steroid hormones that regulate the reproductive function in teleost fish (Amano et al., 1997). FSH and LH are members of the glycoprotein hormone family, which also includes thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG). These hormones consist of a common α subunit and a hormone-specific β subunit. FSH and LH act by binding specific receptors (FSHR and LHR) in the gonads of vertebrates to induce steroidogenesis and gametogenesis (Nagahama et al., 1995).

The cDNA clones and sequences of the GTH subunits have been determined for a number of teleosts, such as European eel (*Anguilla anguilla*) (Querat et al., 1990), killifish (*Fundulus heteroclitus*) (Lin et al., 1992), striped bass (*Morone saxatilis*) (Hassin et al., 1995), channel catfish (*Ictalurus punctatus*) (Liu et al., 1997, 2001), red seabream (*Pagrus major*) (Gen et al., 2000), European sea bass (*Dicentrarchus labrax*) (Mateos et al., 2003), and greasy grouper (*Epinephelus coioides*) (Li et al., 2005), and many studies have shown a temporal pattern of pituitary FSH and LH expression and their circulating levels at different stages of the reproductive cycle (Hassin et al., 2000). In salmonids, plasma FSH levels are high during the early stages of gametogenesis and are believed to control vitellogenesis and spermatogenesis, whereas LH is believed to facilitate gamete maturation, spawning, and ovulation. In contrast, both FSHβ and LHβ mRNA levels of nonsalmonids increase during the spawning season (Kim et al., 2005).

FSHR and LHR cDNA has been isolated from the gonads of several teleosts (Jeng et al., 2007). FSHR and LHR are membrane-bound receptors belonging to the superfamily of G-protein coupled receptors (GPCRs), which contains seven transmembrane domains (7TMD). FSHR and LHR have a large extracellular domain (ECD) that is different from other GPCRs and constitute a subfamily of glycoprotein hormone receptors (GpHRs) that are members of the larger leucine-rich repeat (LRR) containing GPCR (LGR) family. The members of this family have been characterized as containing an ECD with multiple imperfect LRRs flanked by N- and C-terminal cysteine-rich subdomains, and a rhodopsin-like domain of 7TM helices with a C-terminal intracellular tail (Hsu et al., 2000).

The FSHR gene is only expressed in the granulosa cells of the ovary and in the Sertoli cells of the testis, whereas the LHR gene is expressed primarily in the theca and granulosa of preovulatory ovarian follicles

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and in the Leydig cells of the testis (Rocha et al., 2007). Moreover, the FSHR and LHR profiles during oogenesis are consistent with the FSH and LH profiles described in salmonids (Oba et al., 1999a,b).

Black porgy (*Acanthopagrus schlegeli*, Perciformes: Sparidae) is a widely distributed marine protandrous hermaphrodite and a candidate for commercial aquaculture in various parts of Asia, including Korea. Black porgy is functional male for the first two years of life, and then about 70% change sex to female during their third spawning season. To date, despite many studies on sex-change of black porgy (e.g. seasonal GnRH levels, exogenous hormone treatment, LH release and histological analysis) were reported (Chang et al., 1994; Lee et al., 2001, 2004; Du et al., 2005; An et al., 2008), a comprehensive study, investigating the cloning and expression of GTH subunits and their receptors during sex change is lacking for protandrous hermaphrodite fish.

Estradiol-17β (E_2) is involved in the control and release of GTHs in fish (Trudeau et al., 1991; Yen et al., 2002; Lee et al., 2004). Positive E_2 feedback on FSH expression has been reported in primary cultures of pre-pubertal European eel pituitary cells (Aroua et al., 2007) and also, in early and late recrudescent goldfish (*Carassius auratus*) pituitary (Huggard-Nelson et al., 2002). Positive feedback of E_2 on LH expression has been reported in fish, including chinook salmon (*Oncorhynchus tshawytscha*) (Xiong et al., 1994), African catfish (*Mocholkiella paynei*) (Schulz et al., 1995), goldfish (Huggard-Nelson et al., 2002) and black porgy (Lee et al., 2004). Despite the presence of positive and negative feedback of E_2 on GTH expression, its effects are dependent upon species, gonad maturity, hormone concentration, and the duration of exposure to the hormone (Banerjee and Khan, 2008).

The objectives of this study were to 1) first isolate the full-length cDNAs of the GTH subunits and their receptors in black porgy; 2) investigate the effects of E_2 on the brain–pituitary–gonad axis by measuring GTH subunits and GTH receptors mRNA expression following a single injection of E_2 in immature black porgy; and 3) describe the relationship among the GTH subunits, their receptors, and the brain–pituitary–gonad axis of black porgy.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature (51.0±2.3 g, 1-yr old), mature male (220±14.2 g, 2-yr old), sex-changing male (489.2±11.5 g, 3-yr old), and female (948.5±51.6 g, 4-yr old) black porgy. Sexual maturity was determined after excising the gonads defined by the presence of mature ova and sperm. All fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and decapitated prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min) and stored at −80 °C until analysis. Gonad and pituitary samples from black porgy at various stages of sex change and gonadal maturity (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary) were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis.

2.2. E_2 treatment

E_2 was dissolved in ethanol: 0.9% physiological saline (1:1) and then diluted in physiological saline to 1.5 μg/μL. Immature black porgy were anesthetized with MS-222 and injected at a dose of 1 μL/g body mass (BW; 1.5 μg/g). Pituitary, gonad, and blood were collected from three fish at 0, 3, 6, and 9 days following the injection. All of the fish survived the experimental period.

2.3. Total RNA extraction and reverse transcription (RT)

Total RNA from the pituitary and gonads of the E_2-treated black porgy at each stage of gonadal maturation and during sex change was extracted using the TRIzol method according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (2.5 μg) was reverse transcribed in a total volume of 20 μL using an oligo-d$(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.4. Identification of GTH-subunit cDNAs

The primers used for GTHα, FSHβ, and LHβ amplification were designed using highly conserved regions of other teleost fish: GTHα forward primer (5′-CTG GAC TGT CTC TTC TTC TG-3′), GTHα reverse primer (5′-TGT GGT TCC TCA CCC TTA TG-3′), FSHβ forward primer (5′-GGT TGT CAT GGT AGC AGT AG-3′), FSHβ reverse primer (5′-TAG AAG GGC AGA CAT TTG GG-3′), LHβ forward primer (5′-ATG TTG GGT TCC TTC CTG GGA-3′), and LHβ reverse primer (5′-TGC AGA AAT TGG GCT GCA GG-3′). Total RNA was extracted from the pituitary using a TRIzol kit (Gibco/BRL), and PCR amplification was performed using the 2× Taq Premix I (Solgent, Korea) according to the manufacturer's instructions. PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The transformed colony was cultivated in DH5α (RBC Life Sciences, Korea), and the plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoR (Fermentas, Glen Burnie, MD, USA). The GTH-subunit cDNA sequences were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Identification of GTH-receptor cDNAs

The primers used for FSHR and LHR amplification were designed using highly conserved regions of other teleostean fish: FSHR forward primer (5′-AAT CAA GGA GGT GGC AAG TG-3′), FSHR reverse primer (5′-ATC GCT GTC AAC GTG AAC A-3′), LHR forward primer (5′-CCA TCT CCA GAT TCC TCA TG-3′), and LHR reverse primer (5′-TGC ATA CGT AGC AGT AGC AG-3′). Total RNA was extracted from gonads using a TRIzol kit (Gibco/BRL). PCR amplification was conducted using the same method as the GTH subunits, and then the ligation and transformation was also performed using the same method as the GTH subunits.

2.6. Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from the pituitary and gonads using a TRIzol kit (Gibco/BRL). Using 3 μg total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-d$(T)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene).

Gene-specific primers were selected from the PCR products obtained by RT-PCR. For the 3′ RACE, 50 μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 μM 3′ RACE GTHα-specific primer (5′-GTT TTC TCC AGG GAT CGT CCG ATT TAC CAG-3′), 1 μL of 10 μM 3′ RACE FSHβ-specific primer (5′-CAT GAC TGG GCT GAA CAA AGG ACC TGT AAT G-3′), 1 μL of 10 μM 3′ RACE LHβ-specific primer (5′-CAT CTG CAG TGG TCA

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CTG CAT CAC CAA G-3′), 1 μL of 10 μM 3′ RACE FSHR-specific primer (5′-GTC ATA GCG ACT GTA GAC ATA GTC ACC C-3′), 1 μL of 10 μM 3′ RACE LHR-specific primer (5′-CAT GCT CGT CCT CAA TGT AGT CGC TTT CC-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, extension at 72 °C for 1 min, and 1 cycle of final extension at 72 °C for 5 min.

For the 5′ RACE, 50 μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 μM 5′ RACE GTHα-specific primer (5′-CTG GTA AAT CGG ACG ATC CCT GGA GAA AAC-3′), 1 μL of 10 μM 5′ RACE FSHβ-specific primer (5′-CAT TAC AGG TCC TTT GTT CAG CCC AGT CAT G-3′), 1 μL of 10 μM 5′ RACE LHβ-specific primer (5′-CTT GGT GAT GCA GTG ACC ACT GCA GAT G-3′), 1 μL of 10 μM 5′ RACE FSHR-specific primer (5′-CAT CTT TGT GCC GTT GAA GGC GTC ACT TG-3′), 1 μL of 10 μM 5′ RACE LHR-specific primer (5′-CAG GTT GCA CAT GAG GAA TCT GGA GAT GG-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. After PCR, ligation and transformation were conducted using the methods described above.

2.7. Phylogenetic analysis

Phylogenetic analyses have been performed on the amino acid sequences of the full-length GTH subunit and the GTH receptor cDNAs from various fish. Sequences were aligned using the BioEdit Software (Hall, 1999). The sequences we used for comparison are as follows: GTHα: black porgy, red seabream, European sea bass, striped bass, swamp eel (*Monopterus albus*), longtooth grouper (*Epinephelus bruneus*), Hong Kong grouper (*Epinephelus akaara*), rockfish (*Sebastes schlegeli*), pejerrey (*Odontesthes bonariensis*), Mozambique tilapia (*Oreochromis mossambicus*), Nile tilapia (*Oreochromis niloticus*), bastard halibut (*Paralichthys olivaceus*), and Atlantic halibut (*Hippoglossus hippoglossus*). FSHβ: black porgy, red seabream, striped bass, European sea bass, blotched snakehead (*Channa maculate*), three spot gourami (*Trichogaster trichopterus*), convict grouper (*Epinephelus septemfasciatus*), orange spotted grouper (*E. coioides*), bastard halibut, and Atlantic halibut. LHβ: black porgy, yellowfin seabream

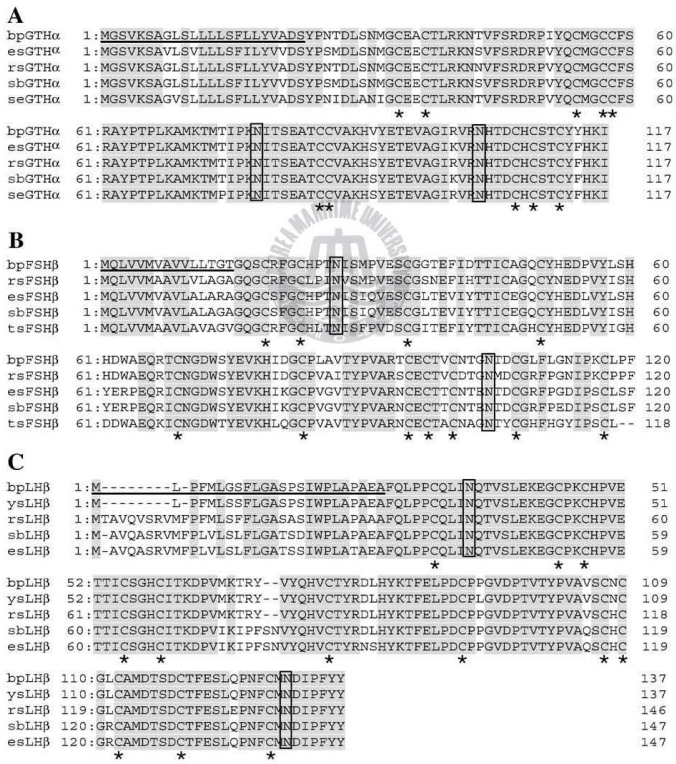

Fig. 1. Comparison of the amino acid sequence of GTHα (A), FSHβ (B), and LHβ (C). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. GenBank accession numbers of the sequences are: GTHα; black porgy (bpGTHα, EF605275), red seabream (rsGTHα, AB028211), swamp eel (seGPα, AF502395), striped bass (sbGTHα, L35071), European sea bass (esGTHα, AF269157), FSHβ; black porgy (bpFSHβ, AY921613), red seabream (rsFSHβ, AB028212), European sea bass (esFSHβ, AF543314), striped bass (sbFSHβ, L35070), three spot gourami (tsFSHβ, AF157630), LHβ; black porgy (bpLHβ, EF605276), yellowfin seabream (ysLHβ, L11722), red seabream (rsLHβ, AB028213), striped bass (sbLHβ, L35096), and European sea bass (esLHβ, AF543315). These sequences are optimally aligned to match identical residues, indicated by the shaded box. The two potential N-linked glycosylation sites are boxed, and cysteine residues of the N- and C-terminal cysteine-rich regions of the ECD are indicated by asterisk. Bold underline indicates signal peptide sequences.

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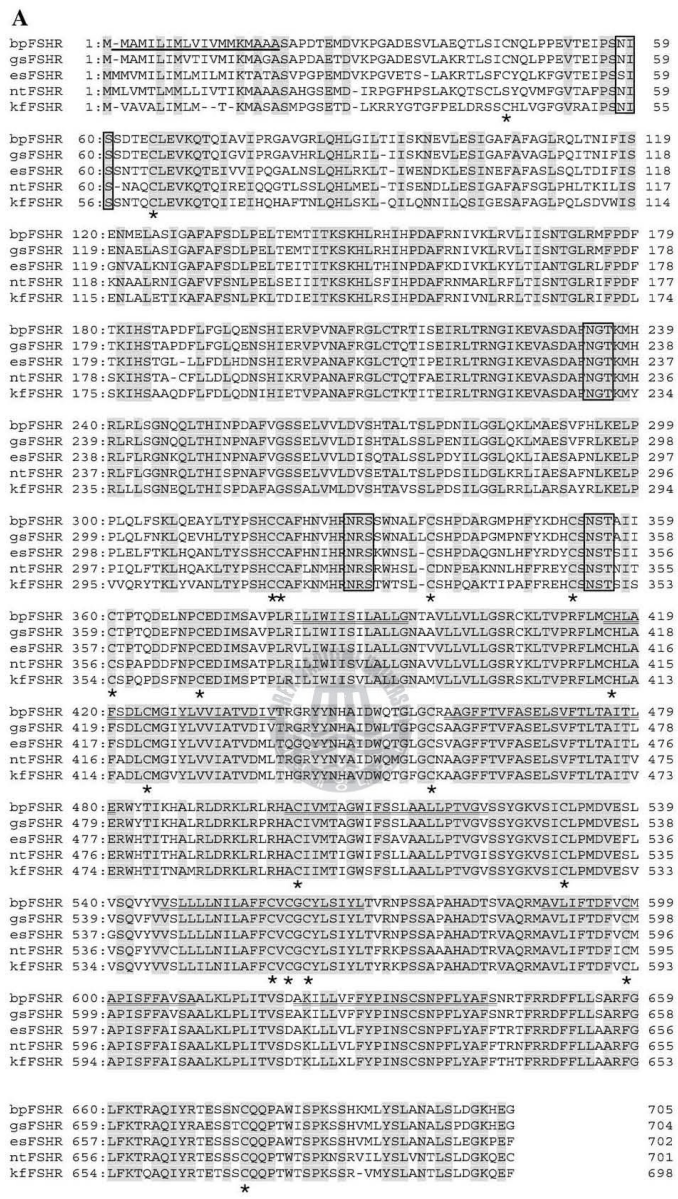

Fig. 2. Comparison of the amino acid sequence of FSHR (A) and LHR (B). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. GenBank accession numbers of the sequences are: FSHR; black porgy (EU095497), gilthead seabream (gsFSHR, AY587262), European sea bass (esFSHR, AY642113), Nile tilapia (ntFSHR, AB041762), and killifish (kfFSHR, AB295490), LHR: black porgy (EU339125), Nile tilapia (ntLHR, AB041763), channel catfish (ccLHR, AF285181), gilthead seabream (gsLHR, AY587261), and Atlantic salmon (asLHR, AJ579790). These sequences are optimally aligned to match identical residues, indicated by the shaded box. The four potential N-linked glycosylation sites are boxed, and cysteine residues of the N- and C-terminal cysteine-rich regions of the ECD are indicated by asterisk. Bold underline indicates signal peptide sequences and double underline indicates TMD.

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B

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bpLHR   1:M----ALREAWLLVALSGVLHVRSCCAFTCPTICRCSADSFHCSGATQLASR-AGPRSVT   55
ntLHR   1:M----ALREVWLLFALSGVLNARSCCAYTCPAICRCTADSFQCSKETQLASR-TGPTSVL   55
ccLHR   1:M----VSRCSVAALLLAVVMRLSRGGAFTCPPICSCTADTLSCTAQTERRTRVPSSTTST   56
gsLHR   1:MRTAPPLLLFL-IV-L-ISCGWKSTSGFVCPRICRCFANTIRCNNVT-QGSALTMEHRDK   56
asLHR   1:MMSISLLLFLFYPSVLLFFGFGCRYASSFVCPGICRCSSNTIRCNNIT-E-KSVPMSERGP   58
                                    *   * *     *

bpLHR  56:RLRLTHLPMKEVPTHAFRELINITTIIEISQSDRVTRIRRHAFLSLHSLAQISLQSINSLR 115
ntLHR  56:RLRLTHLPLKRVPSHAFKELINITTIIEISQSDCITHIQTHAFLSLYSLAQISVQNINSLR 115
ccLHR  57:NLRLAHLPLKEVQSGTFRGLINVSRIEISQSDSIRKIKGEAFLSIHNVIEISILNIIHLS 116
gsLHR  57:RLFLYHLPLHTISSHSFEGLKGVQRIDIAQSVTLETIETLAFNNLLNLSEISIQNTRSLM 116
asLHR  59:RLVLKHLTMSTIASHTFDGLHRVQHIEIGQSVALETIETLAFNNLLDLNEIFIKNTRSLV 118

bpLHR 116:VIEKGAFTDLPKLEYLSISNTGLIHFPDFTTIASL---VPNIILEMADNMRIDIIPANCF 172
ntLHR 116:FIEKGAFADLPKLEYLSISNTGIAHFPDFTTISSL---SPNIILEMADNMEIDIIPANSF 172
ccLHR 117:AMEKGAFTDLPRLRYLSICNTGIRHFPDFSRISSL---GLEFFLDMGDNIQLNNIPANAF 173
gsLHR 117:RIGRGTFNNLPKLRYLSISNTGITVFPDITSIYSLE--PE-FILDIYDNLYLLEIPPNAF 173
asLHR 119:HIARRTFNNLPKLRYLSISNTGITVFPDMTSIHSLEPWNQNFVLDICDNLYLLSIPVNAF 178

bpLHR 173:QGITEEYVDMNLVRNSFTEITAHAFNGTKLNTLNLRDNTYLSDIQEDAFEGATGPSFLDV 232
ntLHR 173:QGITEEYVDMNLVRNGFKEIKSHAFNGTKLNTLVLRDNWYLRNIQEDAFEGATGPTLLDV 232
ccLHR 174:QGISKADMYMNLVRNGFTQIESHAFNGTRLEKLIMKDNWHLIKIHDDAFEGAEGPTLLDV 233
gsLHR 174:IGLTKEYVTMNLYNNGIREIHDHAFNGTKIDKLVLKNNRNLRGIHRDAFKGATGPEVLDV 233
asLHR 179:VGMTTEYTAMNLFNNGIREIQDYAFNGTKINKLVLKNNRNLRVIHREAFKGAVGPRILDV 238

bpLHR 233:SSTALRSLPPKGLRQVRFLKASAAFALKTLPPLESLAELMEAELTYPSHCCAFHTW--RR 290
ntLHR 233:SSTALRSLPPNGLRHVKFLKASHAYALKSLPLLESLAELLEAELTYPSHCCAFHTW--RR 290
ccLHR 234:SSTALKSLPTRGLRGVKVLIARSTPSLKTLPPLDNLAELQEADLTYPSHCCAFHTW--KR 291
gsLHR 234:SATALKKLPAEGLESVLVLFAQSAYALKSLPPLQGLWSLREAHLTYNSHCCALLSWSTHR 293
asLHR 239:SSTAIETLPSHGLNSVVELVARTAYGLKRLPPFRGLGNLQKAHLTYNSHCCALLTWDTHR 298
                                                  **

bpLHR 291:-KQRESA-------LK--------------NSTKFCDLSRTEIEPTA-D-GMNDD----I 322
ntLHR 291:-KQRESA-------LK--------------NLTKFCDLMNTEIDPTADDTSLIND----I 324
ccLHR 292:-NNRETAVFD--R-FK--------------NLTMLCNM-DDQANNMDPSGDDLND----I 328
gsLHR 294:DFTFNPAWNNGS--TSCDESDSTARVQHVIGGSAEGTLPKDLNIFSDADLFVDDESFGDV 351
asLHR 299:DSPINAAQHNGSRPTYCDD-SQSEKFPAGNVDSSDTSLLVEIHG-TNKDV--EDESYGGV 354
                                          *

bpLHR 323:NFQYPDLELDCLNNPFVKCLPKPDASNPCEDLLGFPFLRCLTWIITVFAVAGNLAVLIIL 382
ntLHR 325:NFQYPDLEFDCFSNPFVKCSPKPDAFNPCEDLLGFSFLRCLTWIIMVFAVAGNLAVLVIL 384
ccLHR 329:NFHYPDLEL-CASSSSFKCTPEPDAFNPCEDLLGHTFLRAITWIVTVFALVGNLAVLLVL 387
gsLHR 352:NFHYPELDF-CQTRPTLLCTPEADAFNPCEDIAGFSFLRVAIWFINILAITGNLTVLLVS 410
asLHR 355:DFQYPELGLYCQTRPTLQCTPEADAFNPCEDIAGFSFLRVAIWFINILAIIGNLTVLLIF 414
                  *       *       *

bpLHR 383:LISHSKFTISRFLMCNLAVADLCMGLYLMLIAFMDYHSHHEYYNHATDWQTGPGCGIAGF 442
ntLHR 385:LIGHHKLTVSRFLMCNLAFADLCMGLYLILIAFMDYHSHHEYYNHATDWQTGPGCGIAGF 444
ccLHR 388:LLSHQKLSVSRFLMCNLAFADLCMGFYLLLIAVADYRSRQEYYNHATDWQTGMGCGVAGF 447
gsLHR 411:FTSRNKLTVPRFFMCNLAFADLCIGIYLLMIATVDLRTHGHYSQHAIEWQTGPGCSAAGF 470
asLHR 415:FTSRCKLTVPRFLMCHLAFADFCIGVYLLMIAAVDLHTRGHYSEHAIDWQTGAGCSAAGF 474
                                                          *

bpLHR 443:LTVFASELSVYTLTVISLERWHTITNAMHVNKRLRMHHVAAIMGAGWGFSLLVALLPLVG 502
ntLHR 445:LTVFSSELSVYTLTVISLERWHTITNAMHVNKRLRMHHVTAMMVGGWAFSLLVALLPLVG 504
ccLHR 448:LTVFASELSIYTLTMITLERWHTITHAMQMSRRLRLRHMVTMMAIGWGFSFVIALLPVVG 507
gsLHR 471:LSVFGGELSVYTLSTITLERWHTITNALQVERHLLLTQAASIMAAGWLISLGMGMLPLVG 530
asLHR 475:LSVFGGELSVYTLSTITLERWHTITHALQLEKRLGLAQAAGIMAGGWLICLGMAMLPLVG 534

bpLHR 503:VSSYSRVSICLPMDIDTLGSQVYVVAMLVLNVVAFLVVCYCYVCIYLSVRNPEHSTRHGD 562
ntLHR 505:VSSYSKVSICLPMDIDTLGAQVYVVAVLILNVVAFLVVCYCYICIYLSVHNPEHSTRRGD 564
ccLHR 508:VSSYSKVSICLPMDIETPVSQGYVVAVLVINVAAFLMVCSSYAGIYLSVRNPNVPTRHGH 567
gsLHR 531:VSSYSKVSMCLPMDIETPLAQTFIIIILLFNVGAFIVVCVCYVLIYLAVKNPEVPRRSAD 590
asLHR 535:VSSYSRVSMCLPMDVKTPLAQAFILLLLFFNVGAFLVVCVCYVLIYLAVRNPQFPSRSAD 594

bpLHR 563:TKIAKRMAVLIFTDFVCMAPISFSAISAALRMPLITVSHSKILLILFYPIDSLC-NPFLY 621
ntLHR 565:TKIAKRMAVLIFTDFLCMAPISFFAISAALRMPLITVSHSKILLILFYPINSLC-NPFLY 623
ccLHR 568:TRMAKRMAVLIFTDFLCMAPISFFAISAALHMPLISVSQSKILLILFYPINSLC-NPFLY 626
gsLHR 591:TKMAKRMAVLIFTDFLCMAPISFFAISAAFKVPLITVTNSKILLVLFFPINS-CANPFLY 649
asLHR 595:AKIAKRMAVLIFTDLLCMAPISFFAISAAFKVPLITVTNSKILLVLFFPINS-CANPFLY 653

bpLHR 622:TLFTRAFRRDVCLLLGRCGCCHASADFYRSQTLASHLNSTQKTSTKKTHSLGFYAYHIKM 681
ntLHR 624:TIFTRAFRKDVCLLLSRCGCCNSHADFYRSQTLGSHLTCTQKMSKREPHSLGFYAYHIKM 683
ccLHR 627:TIFTRAFRRDMCLLLSRCGCCHAQAEFYRSQGLLGFTLAPRKKRVRKPHSKNFRAYHVKL 686
gsLHR 650:AIFTKAFRKDAYQLMSALGCCKSKASVHRMNAHCGEKAI--NFGSSYKGSGTAVRLAVME 707
asLHR 654:AIFTKAFRKDVYLLLSNMGCCENKANMYRMKAYCSENLVKSSSGNKGTLICTTQMMDPLP 713
                          **

bpLHR 682:QGCFLNKGST-----                                              691
ntLHR 684:KGCFLNKGTT-----                                              693
ccLHR 687:QGCIFNKAAT-----                                              696
gsLHR 708:GQSHHPKEEGELT--                                              720
asLHR 714:LQSQQLKDDGDLGTI                                              728
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Fig. 2. (*continued*).

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(*Acanthopagrus latus*), red seabream, Atlantic croaker (*Micropogonias undulatus*), striped bass, European sea bass, bamboo leaf wrasse (*Pseudolabrus sieboldi*), orange spotted grouper, longtooth grouper, Nile tilapia, Mozambique tilapia, three spot gourami, and blotched snakehead. FSHR: black porgy, gilthead seabream (*Sparus aurata*), European sea bass, Nile tilapia, killifish, Atlantic salmon (*Salmo salar*), amago (*Oncorhynchus rhodurus*), rainbow trout (*Oncorhynchus mykiss*), Japanese eel (*Anguilla japonica*), and zebrafish (*Danio rerio*). LHR: black porgy, gilthead seabream, European sea bass, Atlantic salmon, rainbow trout, African catfish (*Clarias gariepinus*), zebrafish, grass carp (*Ctenopharyngodon idella*), Japanese eel, channel catfish, and Nile tilapia. The phylogenetic tree was constructed using the neighbor-joining method and the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.8. QPCR

QPCR was conducted to determine the relative expression of GTH-subunits and GTH-receptors mRNA using the total RNA extracted from pituitary and gonads of black porgy. The primers used for the QPCR were GTHα forward primer (5′-AAG ACG ATG ACG ATC CCG AAG-3′), GTHα reverse primer (5′-GTG TGG TTC CTC ACC CTT ATG C-3′), FSHβ forward primer (5′-TGC CAT CCA ACC AAC ATC AGC-3′), FSHβ reverse primer (5′-ATC CTC GTG GTA GCA CTG TCC-3′), LHβ forward primer (5′-ACC AAG GAC CCA GTG ATG AAG AC-3′), LHβ reverse primer (5′-GGG CGG ACA GTC AGG AAG C-3′), FSHR forward primer (5′-CCG ACC CAA GAC GAA CTC AAC-3′), FSHR reverse primer (5′-CCA GCA GGA CCA GAA GCA C-3′), LHR forward primer (5′-GAC GGT GTT CGC CAG TGA G-3′), LHR reverse primer (5′-GCG TTG GTG ATG GTG TGC-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, and annealing at 55 °C for 20 s. Each experimental group was run in triplicate. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold-cycle (Ct) level. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, QPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were as follows: β-actin=94.3%, GTHα=92.8%, FSHβ=96.0%, LHβ=96.5%, FSHR=95.1%, and LHR=92.4%. We performed a melt curve at one temperature to ensure that the primers amplified a specific product.

2.9. Plasma parameter analysis

Plasma E_2 levels were analyzed by radioimmunoassay (RIA) using an E_2 RIA kit (Adaltis, Bologna, Italy).

2.10. Statistical analysis

A one-way analysis of variance followed by a *post hoc* Newman Keuls' test was used to compare differences at a significance level of $P<0.05$. The data were analyzed with the Statistical Package for the Social Sciences version 10.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Identification of GTHα, FSHβ, and LHβ cDNAs

A single PCR product of the expected size (GTHα, 294 base pairs [bp]; FSHβ, 354 bp; and LHβ, 397 bp) was obtained for each subunit. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone the full-length cDNA encoding each of the three GTH subunits.

The full-length GTHα cDNA contained 354 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 117 amino acids. The full-length FSHβ cDNA consisted of 363 nucleotides, including an ORF that was predicted to encode a protein of 120 amino acids. The full-length LHβ cDNA contained 414 nucleotides, including an ORF that was predicted to encode a protein of 137 amino acids. The three GTH subunits contained signal peptides (GTHα, residues 1–23; FSHβ, residues 1–15; and LHβ, residues 1–24), cysteine residues, and a highly conserved N-linked glycosylation site (Fig. 1).

Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that the three GTH-subunit sequences displayed a high similarity with those of other teleosts, and these were compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: GTHα, 95% with red seabream, 92% with swamp eel, 91% with striped bass, and 89% with European sea bass; FSHβ, 81% with red seabream, 75% with European sea bass, 74% with striped bass, and 74% with the three spot gourami; LHβ, 98% with yellowfin seabream, 94% with red seabream, 87% with striped bass, and 86% with European sea bass.

3.2. Identification of FSHR and LHR cDNAs

A single PCR product of the expected size (FSHR, 759 bp; LHR, 471 bp) was obtained for the GTH receptors. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone the full-length cDNA encoding each of the two GTH receptors.

The full-length FSHR cDNA contained 2118 nucleotides, including an ORF that was predicted to encode a protein of 705 amino acids. The full-length LHR cDNA consisted of 2076 nucleotides, including an ORF that was predicted to encode a protein of 691 amino acids.

The FSHR ECD was predicted to contain 380 amino acids, including the signal peptide (positions 1–19), followed by a putative 7TMD (263 amino acids) and an intracellular C-terminal domain (62 amino acids). ClustalX alignment revealed the presence of specific signature sequences (e.g. ^{318}CCAF, ^{480}ERW, ^{593}FTD, and ^{635}NPFLY), which are highly conserved in GpHRs (Vassart et al., 2004). In the areas flanking the LRR region, two cysteines (^{45}C and ^{65}C) and six cysteines (^{318}C, ^{319}C, ^{336}C, ^{353}C, ^{360}C, and ^{370}C) were found that could represent the N- and C-terminal cysteine-rich clusters, respectively. The putative N-linked glycosylation sites identified four motifs at positions ^{58}NIS, ^{234}NGT, ^{326}NRS, and ^{354}NST in the bps of the FSHR. The TMD of the FSHR consisted of 263 amino acids, including seven stretches of 13–23 predominantly hydrophobic residues predicted to form α-helices connected by three intracellular and three extracellular (EC) loops. The conserved ^{480}ERW motif was in the bottom third of the TMD, and the NPXXY motif was also present as a form of ^{633}NPELY, one of the most conserved residues in rhodopsin-like GPCRs. This residue is implicated in receptor activation by switching its interaction between ^{422}D and ^{595}D aspartic residues (Vassart et al., 2004). There were two phosphorylation sites (^{569}T and ^{574}S) in the third intracellular loop and four potential phosphorylation sites (^{646}T, ^{672}S, ^{682}S and ^{686}S) in the intracellular C-terminal domain. Among them, ^{569}T and ^{646}T are potential phosphorylation sites for protein kinase C, and ^{574}S and ^{686}S are potential sites for protein kinase A phosphorylation (Fig. 2A).

Similar to FSHR, the LHR ECD was predicted to contain 361 amino acids, including a signal peptide (positions 1–23), followed by a putative 7TMD (261 amino acids), and an intracellular C-terminal domain (69 amino acids). The LRRs were flanked by 10 conserved cysteines, four of them in an N-terminal cluster (^{26}C, ^{30}C, ^{32}C, and ^{39}C) and six in a C-terminal group (^{282}C, ^{283}C, ^{305}C, ^{333}C, ^{341}C, and ^{351}C). The putative N-linked glycosylation sites identified four motifs at positions ^{71}NIT, ^{198}NGT, ^{299}NST, and ^{375}NLA in the bps of the FSHR. The predicted TMD included seven stretches of hydrophobic residues. The

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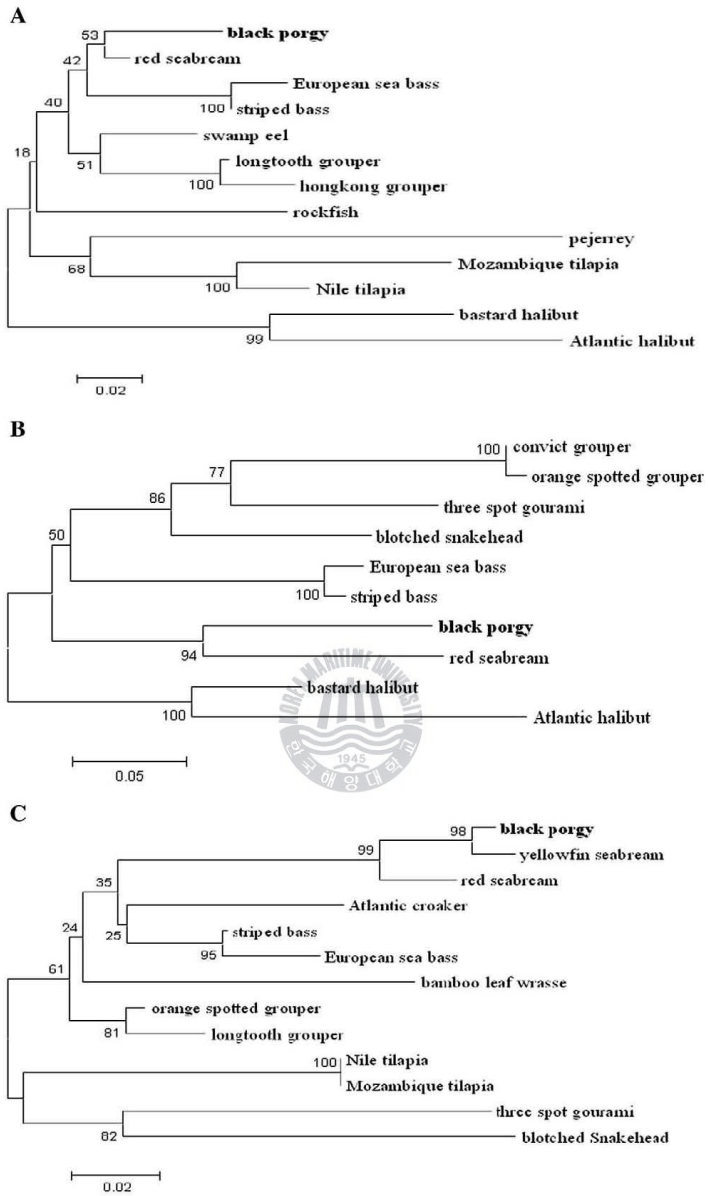

Fig. 3. Phylogenetic tree based on an amino acid alignment for GTH subunits in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: GTHα; black porgy, red seabream, European sea bass, striped bass, swamp eel, longtooth grouper (EF583918), Hong Kong grouper (AY207430), rockfish (AY609078), pejerrey (DQ382280), Mozambique tilapia (AF303087), Nile tilapia (AY294017), bastard halibut (AF268692), and Atlantic halibut (AJ417770), FSHβ; black porgy, red seabream, striped bass, European sea bass, blotched snakehead (AY447038), three spot gourami, convict grouper (AB111457), orange spotted grouper (AY186242), bastard halibut (AB042422) and Atlantic halibut (AJ417768), LHβ; black porgy, yellowfin seabream, red seabream, Atlantic croaker (EF433429), striped bass, European sea bass, bamboo leaf wrasse (AB300391), orange spotted grouper (AF507939), longtooth grouper (EF583920), Nile tilapia (AY294016), Mozambique tilapia (AY541609), three spot gourami (AF157631), and blotched snakehead (AY447037).

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intracellular C-terminal domain consisted of two highly conserved contiguous cysteines (^{641}C and ^{642}C). Similar to the FSHR, the LHR contained highly conserved GpHR signature sequences (e.g. ^{461}ERW, ^{574}FTD, and ^{617}NPFLY). Moreover, the NPXXY motif was also present as a form of ^{617}NPELY, one of the most conserved residues in rhodopsin-like GPCRs (Vassart et al., 2004; Fig. 2B).

Using Blastp, we found that the FSHR and LHR sequences displayed a high similarity to those of other teleosts. The amino acid sequences of the three receptors were compared to those deduced from the cDNA of other teleost species (Fig. 2). The amino acid similarities were as follows: FSHR, 94% with gilthead seabream, 82% with European sea bass, 76% with Nile tilapia, and 74% with killifish; LHR, 84% with Nile tilapia, 62% with channel catfish, 51% with gilthead seabream, and 50% with Atlantic salmon.

3.3. Phylogenetic analysis

The phylogenetic trees of these genes were very similar and generally agreed with the known taxonomic relationships among these species. The phylogenetic analysis indicated the expected relationship among the GTH subunits and GTH receptors. Therefore, we named these genes based on our proposed nomenclature and phylogenetic analysis. The black porgy GTH subunits were most closely related to red seabream (GTHα and FSHβ) and yellowfin seabream (LHβ), which include the Perciformes (Fig. 3). The porgy FSHR was most closely related to gilthead seabream, and the LHR was most closely related to Nile tilapia (Fig. 4).

3.4. Effects of E_2 on GTH-subunit mRNA expression in pituitary

The mRNA of each of the GTH subunits increased significantly after E_2 injection (Fig. 5). The GTHα mRNA increased significantly after 3 days (approximately 5.4-fold that of the control) and then decreased 9 days after the injection (Fig. 5A). The FSHβ mRNA was at its highest after 6 days (approximately 5.9-fold that of the control) and then decreased after 9 days (Fig. 5B). The LHβ mRNA was at its highest after 3 to 6 days (approximately 5.1-fold that of the control) and then decreased after 9 days (Fig. 5C).

3.5. Effects of E_2 on GTH-receptor mRNA expression in gonad

The mRNA of both GTH receptors increased significantly after E_2 injection. The FSHR mRNA was at its highest after 6 days (approximately 5.7-fold that of the control) and then decreased after 9 days (Fig. 6A). The LHR mRNA was at its highest after 6 days (approximately 6.8-fold that of the control) and then decreased after 9 days (Fig. 6B).

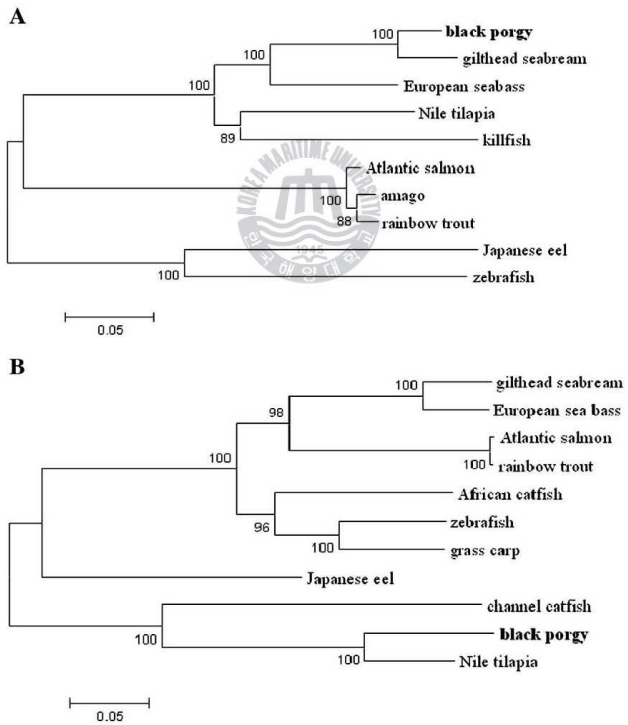

Fig. 4. Phylogenetic tree based on an amino acid alignment for GTH receptors in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: FSHR; black porgy, gilthead seabream, European sea bass, Nile tilapia, killifish, Atlantic salmon (AJ567667), amago (AB030012), rainbow trout (AF439405), Japanese eel (AB360713), and zebrafish (XM_001337064), LHR; black porgy, gilthead seabream, European sea bass (EU282005), Atlantic salmon, rainbow trout (AF439404), African catfish (AF324540), zebrafish (AY714133), grass carp (EF194761), Japanese eel (AY742795), channel catfish, and Nile tilapia.

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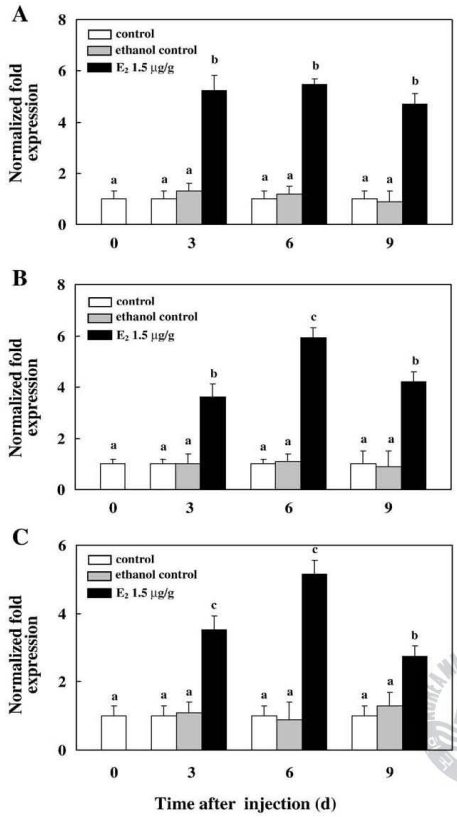

Fig. 5. Expression of GTHα (A), FSHβ (B) and LHβ (C) mRNA in pituitary of black porgy using quantitative PCR by E_2 (1.5 μg/g) injection. 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

3.6. Effects of E_2 on E_2 levels in plasma

The plasma E_2 level was 9.4±0.7 pg/mL at the start of the experiment, increased to a maximum of 260.1±23.54 pg/mL after 3 days, and then decreased to 96.0±13.2 pg/mL after 9 days (Fig. 7).

3.7. Expression of pituitary GTH subunit mRNA during sex change

GTHα mRNA was at its lowest when the fish were immature (Fig. 8A), and FSHβ mRNA was higher in mature males and females but then decreased when the mature gonad became primarily ovary (Fig. 8B). LHβ mRNA was higher in mature males and females (Fig. 8C) than during the other maturation stages.

3.8. Expression of gonadal GTH receptor mRNA during sex change

The FSHR and LHR mRNA was highest in mature males and females than the other maturation stages (Fig. 9).

4. Discussion

In the present study, we first isolated the full-length GTH subunit (GTHα, FSHβ, and LHβ) cDNAs in the pituitary and the GTH receptor (FSHR and LHR) cDNAs in the ovaries of mature females. We studied the effects of E_2 on GTH subunit and GTH receptor mRNA levels in the pituitary and gonads of immature black porgy after the fish were injected with E_2, and investigated interaction between GTH-subunits and GTH receptors during sex change of black porgy.

Using Blastp, we found that the amino acid sequences for the three GTH subunits were highly similar to those of other fish species (GTHα, 89–95%; FSHβ, 74–81%; and LHβ, 86–98%). The cysteine and N-linked glycosylation sites, which are reportedly sites for receptor binding in mammals and fish, were conserved in all three of the GTH subunits of black porgy (Xia et al., 1994; Gen et al., 2000). Similar to the GTHs, we found that the amino acid sequences for the GTH receptors were highly similar to those of other fish species (FSHR, 74–94%; LHR, 50–84%). N-linked glycosylation sites were identified in black porgy GTH receptors and have been linked to binding and expression of specific hormones (Maugars and Schmitz, 2006). The black porgy FSHR and LHR contained the general structural features of a GpHR (Fig. 2). Unlike FSHR, LHR had four cysteines in their N-terminal cysteine-rich region, whereas the FSHR had only two (^{45}C and ^{65}C). This structure allows for the formation of a single disulfide bridge that differs from the location of the two bridges found in the human FSHR (Fan and Hendrickson, 2005), suggesting a difference in the folding of the receptors in this region. There are variable numbers of cysteines in this region of other fish FSHRs (Rocha et al., 2007).

The phylogenetic analysis indicated that the GTH subunits and receptors were closely related to other Perciforms (red seabream GTHα and FSHβ, yellowfin seabream LHβ, gilthead seabream FSHR,

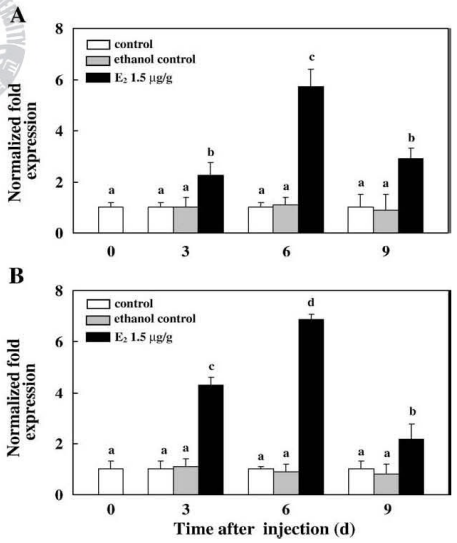

Fig. 6. Expression of FSHR (A), and LHR (B) mRNA in gonads of black porgy using quantitative PCR by E_2 (1.5 μg/g) injection. 2.5 μg of total RNA prepared from gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

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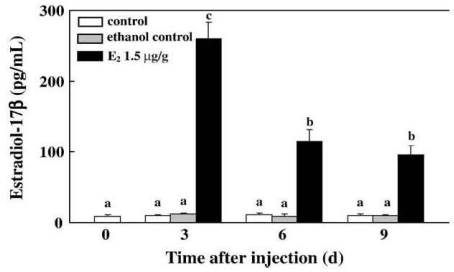

Fig. 7. The plasma estradiol-17β (E_2) levels by estradiol-17β injection in black porgy. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means±SD ($n=3$).

and Nile tilapia LHR). We also found that each of the GTH subunits and receptors was well conserved among other fish species (Figs. 3 and 4).

E_2 was a regulator of the GTH subunits and their receptors as shown by their expression patterns during sex change. The increase of pituitary GTHα mRNA following E_2 injection agreed with the result of a previous study of goldfish (Huggard-Nelson et al., 2002). In agreement with the results of a study of mature grouper (Li et al., 2005), the mRNA expression of the GTH subunits was detected in the pituitary of black porgy at all stages of gonadal maturity, and this expression was relatively higher in mature males and females (Fig. 8). In particular, the GTHα expression increased at mature testis and remained elevated thorough sex change, suggesting that it may be involved with sex change in black porgy; however, it is the common subunit in the pituitary among TSH, FSH, and LH (Salmon et al., 1993).

FSHβ mRNA expression increased following the administration of E_2. This result agreed with a previous study of goldfish that reported increased expression of GTHα, FSHβ, and LHβ (Huggard-Nelson et al., 2002). In the European eel, FSHβ mRNA was increased specifically by E_2, not by testosterone (T) or dihydrotestosterone (DHT) (Aroua et al., 2007). Although FSHβ expression was higher than LHβ in black porgy, we found that the increase was more closely associated with sexual maturation. This result agreed with previous studies demonstrating that FSHβ expression is high during early maturational stages in fish (Gen et al., 2000; Hassin et al., 2000). In mammals, FSH regulation depends not only on steroid hormones but also on gonadal peptides such as activin, inhibin, and follistatin. Activin has been detected in several fish (Pang and Ge, 1999; Yam et al., 1999) and has been shown to regulate FSH in the goldfish pituitary (Yam et al., 1999) and in the zebrafish ovary (Pang and Ge, 1999). Although activin has been detected in the black porgy, FSH expression is dependent on the reproductive stage of the fish (Yaron et al., 2003), so the mechanism of FSH regulation in black porgy remains unclear.

E_2 also increases LHβ mRNA expression in fish (Chang et al., 1994; Xiong et al., 1994; Yen et al., 2002). Also, treatment with exogenous E_2 increases LH levels in goldfish during the spawning season (Huggard-Nelson et al., 2002) and induces sex change in black porgy (Chang et al., 1994, 1995; Lee et al., 2001). It is well established that E_2 is involved with the activation of estrogen response elements (EREs). The presence of EREs on the upstream portion of the LHβ gene has been demonstrated in mammals and fish (Xiong et al., 1994). In chinook salmon, a proximal ERE (pERE) increases LHβ gene transcription by de-repressing the proximal silencer (Xiong et al., 1994), indicating that the stimulatory effects of E_2 may occur, in part, at the level of the LHβ gene in chinook salmon. However, the presence of EREs has not been demonstrated in black porgy. E_2 treatment stimulates seabream GnRH mRNA expression in the preoptic anterior hypothalamic area (POAH), a brain region that projects GnRH neurons to the pituitary (Mohamed et al., 2005). Thus, the positive feedback of E_2 on LH appears to involve GnRH activation, which may stimulate LH synthesis and release.

In mammals, E_2 indirectly stimulates GTH production by upregulating the number of pituitary GnRH receptors (Adams et al., 1981), altering the GnRH receptor mRNA level (Quinones-Jenab et al., 1996), or by increasing GnRH release (Nett et al., 1984). Further studies have demonstrated that the two forms of GnRH present in the European silver eel may be differentially regulated by gonadal steroids (Montero et al., 1995), supporting the view that indirect regulation of GTH production by E_2 may involve GnRH regulation. Treatment with E_2 increases pituitary LH and brain GnRH content (Dufour et al., 1983, 1985). Taken together, these results suggest that E_2 may indirectly regulate GTH production. Further studies are needed to determine the interactions among E_2, GTHs, and GnRHs.

FSHR and LHR expression increased in the gonad following E_2 injection (Fig. 6), which was consistent with pituitary GTH mRNA expression (Fig. 5). The stimulatory effect of E_2 increases GnRH levels in the brain resulting in an increase in the pituitary GTHs (Adams et al.,

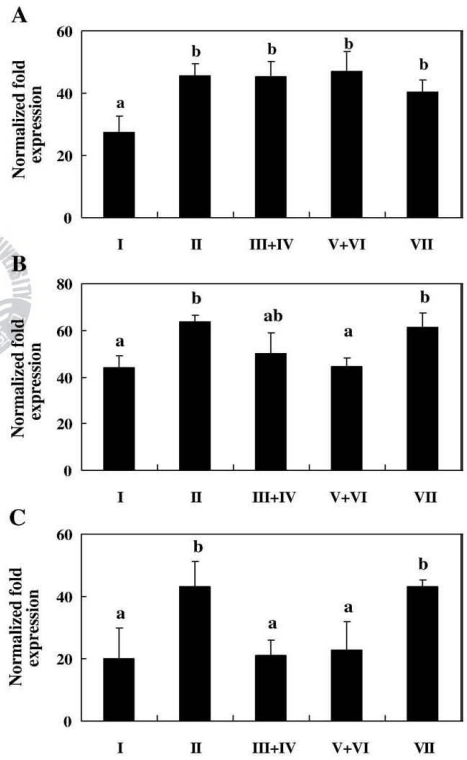

Fig. 8. Expression of GTHα (A), FSHβ (B) and LHβ (C) mRNA in pituitary of black porgy by quantitative real-time PCR. 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III+IV: mostly testicular gonad, V+VI: mostly ovarian gonad, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

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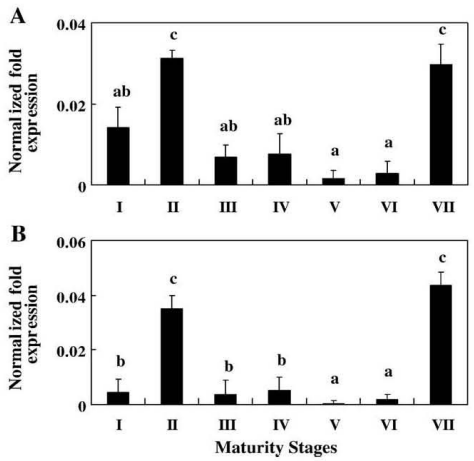

Fig. 9. Expression of FSHR (A) and LHR (B) mRNA in gonads of black porgy by quantitative real-time PCR. 2.5 μg of total RNA prepared from gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III: testicular portion of mostly testis, IV: ovarian portion of mostly testis, V: testicular portion of mostly ovary, VI: ovarian portion of mostly ovary, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

1981), indicating that GTHs are involved in gonadal development by binding to FSHR and LHR (Nagahama et al., 1995).

Although E_2 did not affect FSHR mRNA in Japanese eel (Jeng et al., 2007), E_2 increased pituitary GTHs mRNA as well as FSHR and LHR mRNA expression in gonads of black porgy, demonstrating the involvement of E_2 in gonadal development. However, further studies are necessary to fully establish this association.

In particular, E_2 was found to induce sex change in 1-yr old black porgy by E_2 fed for 7 months, however, the ovary remained at the primary oocyte state (Chang et al., 1994, 1995), and vitellogenic oocytes were observed in 2-yr old black porgy after treatment with E_2 (4–6 mg/kg feed) for at least 5 months (Lee et al., 2000). According to these studies, it is suggested that E_2 induces sex change of black porgy. We found that the maximum plasma E_2 level (3 days; 260.1 ± 23.54 pg/mL) following E_2 injection was lower than that of mature female black porgy (705.6±70 pg/mL) during sex change determined in our previous study (An et al., 2008). These data suggest that the plasma E_2 concentration attained following the exogenous administration of E_2 did not affect sex change but only temporarily increased plasma levels in immature black porgy. Therefore, these results combined with these previous reports suggest that E_2 is involved in ovarian maturation as well as the male-to-female sex change in black porgy.

The mRNA of the GTH subunits and GTH receptors increased in mature male and female black porgy. The increased FSHR and LHR mRNA implies that pituitary-stimulated FSH and LH release is involved in gonadal development.

In conclusion, GTH-subunit and GTH-receptor cDNAs were isolated from mature female black porgy, and the mRNA expression of the genes was investigated following E_2 injection of immature fish. We also compared the mRNA expression at each stage of the sex change process. We found that E_2 stimulated GTH-subunits mRNA in the pituitary and GTH-receptors mRNA in the gonads. Our results showed a high level of expression in the immature gonads, indicating that E_2 affects the brain–pituitary–gonad axis and leads to gonadal development. Additional studies, including those with different doses and treatments during gonadal development, will be necessary to fully identify the roles of E_2 in maturation. The results of this study can be used to elucidate the endocrinological mechanism and relationship between E_2 and the GTH subunits in black porgy.

Acknowledgements

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V. Conclusion

In this study, to identify the mechanism of sex-change in the protandrous black porgy, *Acanthopagrus schlegeli*, I compared the expression of the sex-change related genes by quantitative real-time PCR (QPCR) in the gonads and pituitary during sex-change process of black porgy from male to female. The expression of sGnRH, sbGnRH, and cGnRH-II were found to be high in mature testis and ovary. ERα and ERβ1 were high in the mature testis and ovary, but ERβ2 was higher in the mature ovary. Also, GTHα, FSHβ, and LHβ were high in pituitary of mature male and mature female, and FSHR and LHR were high in mature testis and mature ovary. In histological study, I observed the vitellogenic oocytes and increase of E_2 level in plasma in the mature female of black porgy. In addition, to investigate the action of GnRH and E_2 in the black porgy, I injected with GnRHa and E_2 in immature black porgy. The expression of GTH subunits and GTH receptors and E_2 level in plasma were increased after GnRHa and E_2 injection.

Therefore, it is concluded that these genes were involved in the sex-change of black porgy, play important roles in the regulation of hypothalamus-pituitary-gonad axis of black porgy.

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